REPRODUCTIVE PHYSIOLOGY OF THE

TASMANIAN DEVIL (*SARCOPHILUS HARISII*)
AND
SPOTTED-TAILED QUOLL (*DASYURUS MACULATUS*)

by

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This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due acknowledgement is made in the text.

Heather Hesterman

October 2008

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DEDICATION

in Memory of

Edward "Ted" Rudolph Hesterman, formerly of CSIRO

for scientific aspiration

and;

Dr Irynej Joseph Skira, formerly of DPIWE

for mentoring and sage advice

ABSTRACT

The Tasmanian devil (*Sarcophilis harrisii*) and the spotted-tailed quoll (*Dasyurus maculatus*) are the world's largest extant carnivorous marsupials. These two closely related dasyurid species coexist only on the island of Tasmania, and both are listed as Threatened.

The aim of this study was to develop a definitive understanding of the reproductive processes of these large, sympatric dasyurids to gain insight into how aspects of the physical and social environment shape evolutionary life history strategies. Although both species are solitary, the devil is gregarious and relatively abundant in comparison with the spotted-tailed quoll. Furthermore, in Tasmania *D. maculatus* experiences a high degree of interspecific competition for food - the ultimate factor influencing breeding timing and synchrony. I hypothesised that these differences in population density, the level of sociality, and access to nutritional resources would be reflected in the species' reproductive biology, including the duration of breeding season, mechanism of ovulation, synchrony of oestrus, fecundity and lifetime reproductive effort.

The ovarian and testicular cycles were characterised in captive and free-ranging devil and spotted-tailed quoll populations, and breeding seasonality was compared with patterns found in other dasyurids.

In female devil and spotted-tailed quoll, longitudinal endocrine profiles revealed a biphasic pattern of plasma progesterone, with a characteristic pro-estrous pulse during the follicular phase (FP), occurring up to several weeks prior to onset of the luteal phase (LP). The patterns of faecal metabolites (20α-OH-pregnanes, 20-oxo-pregnanes) were positively correlated with fluctuations in plasma progesterone. Mean duration of the oestrous cycle (FP + LP) was ~32 days for devils and ~38 days for spotted-tailed quolls. Significant differences between the pattern of progestagens and estrogens concentrations during the pregnant and non-mated oestrous cycle, suggest maternal recognition of pregnancy in the devil.

Changes in pouch appearance during oestrous have been documented as an indicator of breeding condition in a number of dasyurids. Pouch condition of female devils and

quolls was assessed based on size, colour and secretions, and found to accurately reflect reproductive status. The stage of pouch development was also correlated with underlying changes in development of the reproductive tract.

In male devils peak androgen concentrations occurred between December - March (austral spring/summer). There was no seasonal change in scrotal dimension or size/mass of the testes, epididymides or prostate. In devils, an extended period of spermatogenesis was apparent: sperm were produced from November until August. In spotted-tailed quolls, peak androgen concentrations were recorded between April - July (austral autumn/winter). Spermatogenesis in the spotted-tailed quoll began by January, and sperm were produced from April until August. Differences in the annual timing of breeding in these two species is likely caused by differing responses to photoperiod – with the devil cued by increasing day length during spring, and the quoll stimulated by decreasing photoperiod in autumn.

Although breeding was not tightly synchronised within either devil or spotted-tailed quoll populations, late lactation and weaning usually occurred during the optimal period of late spring/summer. Findings indicate that timing of reproductive events can be relaxed in species where ecological and reproductive attributes permit a level of flexibility. In devils and spotted-tailed quolls these are large body size, generalist flesh-eating diet, facultative polyoestry and variation in the length of lactation. This study confirms the devil is facultatively polyoestrous and can breed at 12 months of age, therefore can now be classified alongside the spotted-tailed quoll as having a strategy III life-history.

The fundamental information gained on the reproductive biology of the largest dasyurids will be applied to improve and assist *in situ* and *ex situ* conservation and management of these threatened marsupial species.

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PREFACE

The main body of this thesis comprises five chapters prepared as manuscripts for publication in peer-reviewed scientific journals. Three of these papers are already published, and a fourth is currently in press. The manuscripts are provided here in their entirety including their abstracts and references; independent formatting styles of the journals have also been retained, notable as discrepancies between different English languages (US or UK).

I am the senior author on all publications, all of which also include my primary supervisor, Associate Professor Susan Jones as co-author. Additional co-authors are my field supervisor Dr Menna Jones, and Associate Professor Franz Schwarzenberger of the University of Veterinary Medicine Vienna who contributed through analyses of female faecal samples.

Publications or prepared manuscripts form the body of this thesis:

Chapter 2
Hesterman H, Jones SM, Schwarzenberger F. (2008). Plasma and fecal steroid monitoring of ovarian cycles in large dasyurids: I The Tasmanian devil (*Sarcophilus harrisii*). General and Comparative Endocrinology 155: 244 - 254

Chapter 3
Hesterman H, Jones SM, Schwarzenberger F. (2008). Plasma and fecal steroid monitoring of ovarian cycles in large dasyurids: II The Spotted-tailed Quoll (*Dasyurus maculatus*). General and Comparative Endocrinology 155: 234 – 243.

Chapter 4
Hesterman H, Jones SM, Schwarzenberger F. (2008). Pouch condition is a reliable indicator of breeding condition in the Tasmanian devil (*Sarcophilus harrisii*) and spotted-tailed quoll (*Dasyurus maculatus*). Journal of Zoology, London 275: 130 – 138.

Chapter 5
Hesterman H, Jones SM. (2008) Longitudinal monitoring of plasma and faecal androgens in the male Tasmanian devil (*Sarcophilus harrisii*) and the spotted-tailed quoll (*Dasyurus maculatus*). Animal Reproduction Science. In press, accepted October 2007 doi:10.1016/j.anireprosci.2008.05.071.

Chapter 6
Hesterman H, Jones SM, Jones ME. Flexibility in reproductive seasonality and synchrony in the two largest marsupial carnivores. *In prep.*

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CHAPTER 1
GENERAL INTRODUCTION

The purpose of this general introduction is not to provide a comprehensive review of marsupial reproductive physiology or its evolution, as these areas have already been well covered (see: Tyndale-Biscoe 1984; Lee and Cockburn 1985; Tyndale-Biscoe and Renfree 1987; Cockburn and Johnson 1988; Tyndale-Biscoe 2005), and pertinent information will be detailed in the following chapters. Rather, the purpose is to provide some background knowledge and to identify some gaps in our understanding that lead to the inception of this PhD project. To place this study in context, I begin by providing a synopsis of past research on marsupial breeding biology, followed by a review of current knowledge of the reproductive biology of the Family Dasyuridae, prior to introducing the study species and outlining objectives of this research.

1.1 Marsupial Reproductive Biology

Around 500 years ago when American and Australian marsupials were first described, it was the presence of a pouch and tiny young that invoked fascination. By the early 1900s the anatomy and embryology of two polyprotodont species: the north American opossum (*Didelphis virginiana*), and an Australian carnivorous species – the eastern quoll (*Dasyurus viverrinus*), had been pioneered (see Tyndale-Biscoe & Renfree 1987 for review). Reproductive endocrinology of marsupials was pioneered in the 1920s with further research on the north American opossum (Hartman, cited in Tyndale-Biscoe 1984); however, it was the discovery of delayed implantation in macropods in the mid-1950s that aroused special interest in the hormonal control of reproduction, and stimulated the first experimental work on diprotodonts (Tyndale-Biscoe 1984). This advance permitted the oestrous cycles of polyprotodonts and diprotodonts to be described in the 1960s - revealing two discrete patterns based on the duration of pregnancy relative to the length of oestrous cycle (Sharman *et al.* 1966; Tyndale-Biscoe and Renfree 1987).

The 1970s marked an important era with the development of plasma assays for steroid/protein hormones. However, despite these new technologies, our understanding of the hormonal control of reproduction in more than a handful of species remained

disconcertedly limited by the early 1980s (Tyndale-Biscoe 1984). Soon afterward, further technological advances in endocrine techniques – including non-invasive methods (urinary and faecal steroids), generated this essential information for a range of male and female mammals (Monfort 2003) including other marsupial species (Tyndale-Biscoe and Renfree 1987). Non-invasive steroid measurement has since been widely applied to monitor reproduction and stress in free-ranging and captive eutherian species (reviews in: Lasley and Kirkpatrick 1991; Schwarzenberger *et al.* 1996; Monfort 2003), but despite the benefits and potential of this practical technique, few researchers have applied this technique to marsupials (Hamilton *et al.* 2000; Stead-Richardson *et al.* 2001; Paris *et al.* 2002; Bradshaw *et al.* 2004; Woodd *et al.* 2006).

As our specific knowledge of marsupials has improved, there has been an important shift away from simply describing the various physiological, ecological or behavioural patterns of this intriguing group, toward consideration of the interplay of such features, and how such studies can contribute to a broader understanding of mammalian biology (Russell 1982; 1984; Lee and Cockburn 1985; Tyndale-Biscoe and Renfree 1987; Cockburn and Johnson 1988; Cockburn 1989). For marsupials, this trend toward a more holistic approach began in the 1970s, when researchers began to explore the evolutionary ecology driving various reproductive strategies (Lee and Cockburn 1985; Tyndale-Biscoe and Renfree 1987). This approach has demonstrated how reproductive patterns are shaped not only by habitat, but also modified by diet and the related feature of body size (Tyndale-Biscoe 1984; Lee and Cockburn 1985). Therefore, generalities cannot necessarily be entertained; because, even within Families, species may exhibit a range of different reproductive strategies.

There is also a clear and pressing conservation need to continue to improve our knowledge of breeding biology in a wider range of species (Wildt *et al.* 2003). Mammalian research tends to focus on generating comprehensive information on a limited number of relatively common species (Bronson 1989; Temple-Smith 2003; Wildt *et al.* 2003); and for marsupials, our apparent preoccupation with macropods has continued (Tyndale-Biscoe and Renfree 1987). Detailed studies of particular species are undoubtedly necessary to gain a thorough knowledge of reproductive processes and to further modern directions in this field, such as assisted breeding techniques including

artificial insemination and embryo transfer (Holt 1994; Hinds *et al.* 1996; Temple-Smith 2003; Andrabi and Maxwell 2007). Unfortunately, that technology is not readily transferred to wildlife, and remains a somewhat idealistic goal that may be detrimental to a broader understanding of reproductive biology and more realistic approaches to conservation (Temple-Smith 2003; Wildt *et al.* 2003).

Information on male breeding biology is a necessary and complementary step to understanding species' reproductive patterns, but this area has remained largely overlooked compared to the wealth of research on the female marsupial. It is suggested this oversight has resulted from the males' closer similarity to eutherians (Tyndale-Biscoe and Renfree 1987), because, apart from marked differences in sperm morphology and function (e.g. Hughes 1965; Setchell and Carrick 1973; Breed *et al.* 2003), differences in the anatomy of the genital tract are subtle, and generally the form and function of these structures follow the eutherian plan. Further research on males is warranted to extend our understanding of reproductive processes, and permit a full appreciation of breeding strategies within marsupials.

1.2 The Dasyuridae

The Dasyuridae are a diverse family of carnivorous marsupials represented by more than 60 extant species, found exclusively in Australia and Papua New Guinea. Dasyurids occupy vastly different climatic zones and habitats from arid areas and grasslands, to temperate and tropical rainforest, and range in body size from less than 20 g to over 10 kg (Strahan 2005). Accordingly, they are characterised by a suite of reproductive strategies based on parameters including seasonality, age at sexual maturity and the frequency and duration of male and female reproductive effort (Lee *et al.* 1982; Lee and Cockburn 1985). Dasyurids are short-lived compared to eutherians of similar size, and as a result, have an abbreviated window for reproduction (Cockburn 1997). Mortality is an important predictor of variation in mammalian life histories (Promislow and Harvey 1990) - epitomised by the evolution of semelparity in dasyurids such as *Antechinus* and others, characterised by abrupt, post-mating mortality of males (Dickman and Braithwaite 1992; Dickman 1993; Cockburn 1997).

Most dasyurids are seasonal breeders and ecologically monoestrous, rearing a single litter each year. This is due to a limited period suitable for reproduction in most Australian regions, and the relatively long period of lactation (Lee *et al.* 1982). Exceptions are some inhabitants of arid zones (*e.g. Sminthopsis, Dasyuroides byrnei*) or tropical regions (Murexia = New Guinea *Antechinus sp.*), where opportunistic strategies permit the production of two or more litters annually (McAllan 2003). However, nearly all dasyurids are facultatively polyoestrous and will return to oestrus if conception fails or a litter are lost during the breeding season (Tyndale-Biscoe and Renfree 1987). This feature is typical of marsupials, but particularly important for dasyurids because they have a short reproductive life span (Lee *et al.* 1982).

1.3 Reproduction of the Female Dasyurid

There have been a series of early, comprehensive studies on the eastern quoll (O'Donoghue 1911; 1912; Hill and O'Donoghue 1913; Hill and Hill 1955) and considerable focus on Antechinus (Woolley 1966; Selwood 1980; Selwood 1985; Taggart and Temple-Smith 1991). Despite the interesting features of this Family, the reproductive biology of few other dasyurids has been studied in any detail, and data on many species are still unavailable (Krajewski *et al.* 2000). The last comprehensive review on reproductive physiology of marsupials was conducted by Tyndale-Biscoe and Renfree in 1987. Since that time additional efforts to extend this database are apparent (Hinds 1989; Hinds and Selwood 1990; Woolley 1990a; Selwood and Woolley 1991; Taggart and Temple-Smith 1991; Woolley 1991b; 1991a; Millis *et al.* 1999; Cruz *et al.* 2001; Stead-Richardson *et al.* 2001; Woolley 2003), although research has again centralised on members of the Genera *Antechinus* and *Sminthopsis*. Enough basic information has been generated on the reproductive biology of dasyurids to provide a useful level of comparative knowledge. Dasyurids conform to the basic reproductive pattern seen in non-macropod marsupials (Tyndale-Biscoe and Renfree 1987). The luteal phase occupies around 60% of the estrous cycle and following parturition, subsequent follicular activity is suppressed by lactation. Like most marsupials, ovulation occurs spontaneously with the main hormones oestradiol and progesterone acting on the female reproductive system in a manner analogous to that described for eutherian mammals (Hinds *et al.* 1996). The pattern of follicular growth and maturation

at oestrus is also similar between groups; the primary difference is that in marsupials, the corpus luteum is autonomous and does not require luteotrophic support from the pituitary, placenta or uterus after formation (Hinds 1990). In non-macropod marsupials, the life of the corpus luteum is not affected by pregnancy; and in all species, the duration of the pregnant and non-pregnant oestrous cycle are equivalent. These phases are also physically and physiologically indistinguishable, and females undergo the same developmental changes of the genital tract and mammary glands (Tyndale-Biscoe and Renfree 1987, but see: Harder and Fleming 1981; Renfree 2000), as documented for a number of eutherian mammals (Rowlands and Weir 1984).

In dasyurids, the oestrous cycle lasts between three to eight weeks depending on the species (Tyndale-Biscoe and Renfree 1987). This information has largely been determined through basic techniques such as monitoring of breeding events, studies of the histological appearance of the reproductive tract and assessment of vaginal cytology. There have been remarkably few additional studies of reproductive endocrinology in dasyurids over the last 20 years (Hinds 1989; Hinds and Selwood 1990; Millis *et al.* 1999; Stead-Richardson *et al.* 2001). In smaller dasyurids, changes in body mass can also be used to monitor the oestrous cycle (Tyndale-Biscoe and Renfree 1987), and have been correlated with plasma progesterone concentrations (Fletcher 1985b). Development of the pouch area during the breeding season is also commonly observed in dasyurids, and may be used in association with other techniques to detect onset of reproductive activity (Woolley 1966; 1974; Tyndale-Biscoe and Renfree 1987), although rigorous validations of this technique have not been performed.

1.4 Reproduction of the Male Dasyurid

Similar to other male mammals (Lincoln 1981), in marsupials seasonal changes associated with the breeding season include an increase in plasma androgen (testosterone or 5α-dihydotestosterone) concentrations (Tyndale-Biscoe and Renfree 1987). Measurement of androgen concentrations have been conducted for some dasyurids, in particular study of hormonal events associated with breeding in semelparous species (Tyndale-Biscoe and Renfree 1987; Schmitt *et al.* 1989; Millis *et al.* 1999; Bradley 2003). A corresponding increase in testes size and/or mass of the epididymides associated with commencement of spermatogenesis, as well as enlargement of the

accessory organs (prostate and Cowpers' gland), is apparent in seasonal breeders (Tyndale-Biscoe and Renfree 1987). The rise in androgen concentrations prepares males behaviourally and physiologically for the mating season, and typically these changes commence in advance of the breeding period. Heightened androgen concentrations fuel the aggressive rut-like behaviour observed in male dasyurids such as *Antechinus*; and together with a concomitant increase in plasma cortisol concentrations, these lead to the stress-related post-mating death (reviewed in Bradley 2003).

Environmental cues stimulate sperm production early in the season to ensure fertility coincides with the onset of breeding condition in the female, and spermatogenesis is usually maintained beyond the annual period of oestrous activity in the population (Bronson 1989; Tyndale-Biscoe 2005). This pattern is observed in iteroparous dasyurids and other marsupials (Tyndale-Biscoe and Renfree 1987; Woolley 1990b). In semelparous dasyurids, however, the timing of spermatogenic failure is earlier and males rely instead on epididymal sperm reserves - potentially as an energy conservation measure (Taggart *et al.* 2003). Spermatogenic failure is suggested to have arisen because of male die-off in Antechinus, and is an adaptation that prevents highly elevated levels of androgens associated with the rut from interfering with sperm production (Cockburn 1997). Energetic costs associated with breeding can be considerable, from production of gametes and seminal plasma, to territory defence and procurement of mates (Tyndale-Biscoe 2005). Reproductive strategies of the male have therefore evolved to offset such costs by ensuring timing of breeding coincides with that of the female, and they are responsible for maintaining synchrony between the sexes (Sadlier 1969; Tyndale-Biscoe 2005).

1.5 The Tasmanian Devil and Spotted-tailed Quoll

The Tasmanian devil (*Sarcophilus harrisii*) and spotted-tailed quoll (*Dasyurus maculatus*) are distinguished from other dasyurids by their diet, which consists predominantly of vertebrate flesh, and their substantial body size (maximum 7 - 12 kg, respectively: Strahan 2005). Devils became extinct on the Australian mainland ~ 400 – 5000 years ago, but spotted-tailed quoll populations continue to persist in fragmented zones within the south-eastern states and far north Queensland (Jones *et al.* 2003). These two species now coexist only on the island of Tasmania, alongside the smaller,

insectivorous eastern quoll, and are sympatric across much of their range (Jones 1997). Although the devil and spotted-tailed quoll are solitary by definition (Russell 1984), they exhibit extremes in terms of sociality and organisation. Spotted-tailed quolls occupy large home ranges and females are territorial (Belcher and Darrant 2004; Claridge *et al.* 2005; Glen and Dickman 2006), whereas devils have smaller, overlapping home ranges and form temporary feeding associations (Buchmann and Guiler 1977; Pemberton and Renouf 1993). Research has focused on various other aspects of the ecology of the devil and spotted-tailed quoll (Guiler 1970b; Manserg 1984; Green and Scarborough 1990; Pemberton and Renouf 1993; Jones and Barmuta 1998; 2000; Belcher and Darrant 2004; Körtner *et al.* 2004), but fundamental information on their breeding biology has remained lacking.

Basic life history variables are available for the devil and for the spotted-tailed quoll (Fleay 1952; Guiler 1970a; Settle 1978; Manserg 1984; Belcher 2003); and past research also includes some details of reproductive anatomy, oogenesis, sperm structure and embryology - mainly for the devil (Flynn 1910; 1911; O'Donoghue 1912; Flynn 1939; Pearson and De Bavay 1953; Hughes 1982). Both species are seasonal breeders. Mating and births occur in March/April for devils and June/July for spotted-tailed quolls, and both species wean their young in spring (Fleay 1935; 1940; Green 1967; Guiler 1970a; Belcher 2003). Dasyurids typically have a well regulated annual breeding period (McAllan 2003), yet there is some intriguing evidence for out-of-phase breeding in both the devil and spotted-tailed quoll (Guiler 1970a; Green and Scarborough 1990; Körtner 2006). Study of the reproductive patterns of these ecologically distinct dasyurid species will provide a useful comparison with other marsupial and eutherian mammals, particularly for developing our understanding of the evolution of breeding strategies. Furthermore, this research will have a practical, conservation application for monitoring and management of *in situ* and captive populations.

Carnivorous marsupials are vulnerable to extinction events for several reasons, including their characteristically rapid life history which imposes natural constraints on reproductive output (Cockburn 1997; Jones *et al.* 2003). At the onset of this study, the status of the devil and spotted-tailed quoll in Tasmania was considered secure, but both species are now listed on state and federal Threatened species schedules

(www.dpiw.tas.gov.au/threatenedspecies). Spotted-tailed quolls are naturally rare across most of their range, and primarily endangered by habitat loss (Jones *et al.* 2003). Devils became extinct on the Australian mainland ~ 400 – 5000 years ago (Jones *et al.* 2003) but remained widespread and common in Tasmania until very recently; with the species currently facing serious risk of extinction from a rapidly spreading, and contagious fatal disease (Hawkins *et al.* 2006; Pearce and Swift 2006; McCallum *et al.* 2007) (Hawkins *et al.* 2006; Pearce and Swift 2006). For both species, there is an obvious necessity to maintain self-sustaining captive 'insurance' populations. This is particularly challenging because of their characteristically short reproductive life span (1 – 3 yrs) and low breeding output (Jones *et al.* 2003). Zoos have had limited breeding success with both species, and a lack of information on their reproductive biology is implicated as the main cause (Williams 1990; Carnio 1993; Jackson 2003a).

1.6 Study Aims

The central objective of this study was to establish a comprehensive understanding of reproductive processes in the sympatric Tasmanian devil and spotted-tailed quoll – the world's largest, vertebrate-feeding carnivorous marsupials and only dasyurids, to elucidate the role of the physical and social environment in evolutionary shaping of life history strategies.

Specifically, I aimed to characterise the female and male reproductive cycle of the Tasmanian devil and spotted-tailed quoll; and to determine the onset and timing of key reproductive events including puberty, oestrus, spermatogenesis, births and fecundity in free-ranging populations. A secondary intent was to develop alternative, non-invasive techniques for *in situ* and *ex situ* monitoring of reproduction. These aims were addressed through a longitudinal study of captive individuals, and field studies of wild devil and spotted-tailed quoll populations.

For females, techniques included measurement of sex steroids in plasma (progesterone) and faeces (progestagens and oestrogens), as well as an assessment of vaginal smears, changes in pouch appearance, and gross anatomy of the reproductive tract during the oestrous cycle. Chapters 2 and 3 provide information on the reproductive endocrinology of captive devils and spotted-tailed quolls, respectively. In Chapter 4, I document

characteristic changes in pouch appearance correlated with breeding status as determined by hormone monitoring and vaginal cytology. Morphometric and demographic data were also collected from female and male devils and spotted-tailed quolls to further investigate changes associated with breeding; and to determine the onset and timing of key breeding events including puberty, birth, pouch exit and weaning.

For male devils and quolls, physical and physiological data was collected to monitor and describe changes associated with breeding (Chapters 5 and 6: captive and wild populations, respectively). Reproductive activity was assessed through analysis of plasma and faecal androgens (Chapters 5, 6) and assessment of changes in the size and/or mass of the testes, epididymides and prostate (Chapter 6). For wild populations, stages of spermatogenesis were evaluated through histology of the testes and epididymides.

Chapter 6 documents the seasonality of breeding in free-ranging devil and spotted-tailed quoll populations. I predicted that the marked difference in diet and associated larger body size of the Tasmanian devil and spotted-tailed quoll compared to other members of the Family, would be reflected in differences in their reproductive patterns and strategies. Furthermore, I anticipated that differences in density and socio-spatial organisation between the devil and spotted-tailed quoll would also influence specific features of each species' breeding biology such as the mechanism and pattern of oestrous, and timing/synchrony of reproductive events in the population.

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CHAPTER 2

PLASMA AND FECAL STEROID MONITORING OF OVARIAN CYCLES IN THE TASMANIAN DEVIL (*SARCOPHILUS HARRISII*)

2.1 Abstract

There is a strong body of knowledge on the reproductive endocrinology of macropods, but little detailed information is available on the hormonal control of reproduction in other marsupials. This study used plasma and fecal sex steroid monitoring to characterize the estrous cycle of the largest extant dasyurid—the Tasmanian devil (Sarcophilus harrisii). A pro-estrous pulse in plasma progesterone (1.33 ± 0.2 ng/ml) occurred several weeks prior to onset of the luteal phase (LP), resulting in a characteristic biphasic pattern during the estrous cycle. This brief, pro-estrous progesterone pulse was associated with a predominantly cornified vaginal smear, and copulation in females paired with males. Mean luteal progesterone concentrations (5.28 ± 0.8 ng/ml) were sustained and peaked around day 15 from luteal onset; thereafter, concentrations declined precipitously and returned to baseline around day 25. Females that did not produce young returned to estrus after 33.7 ± 5.9 days. Fecal 20a-OH-pregnanes analyzed in a pregnanediol assay (PgD) were excreted in consistently higher levels than 20-oxo-pregnanes, but the pattern was similar for the two metabolites, and significantly correlated with fluctuations in plasma progesterone. Fecal total estrogen concentrations were highest during the follicular phase (FP) and accompanied a pro-estrous pulse in fecal progestagens. The mean duration of the estrous cycle was ~32 days, with a FP of around 14 days (range 8–23 days), and a luteal phase of around 18 days (range 12–25 days). There were no differences in the length of the LP between mated and non-mated cycles. Gestation length was 17.9 ± 1.0 days (range 14–22 days). Fecal steroid monitoring revealed significant differences between the pattern of progestagens and estrogen concentrations during the pregnant and non-mated estrous cycle, suggesting maternal endocrine recognition of pregnancy in the Tasmanian devil.

2.2 Introduction

The importance of developing knowledge of the reproductive endocrinology of marsupials as a basis for comparative research is well recognised (Tyndale-Biscoe, 1984). There are two primary patterns of reproduction in marsupials, the basic pattern common to most species (*e.g.* dasyurids, possums) and the macropod pattern found in most wallabies and kangaroos (Tyndale-Biscoe and Renfree, 1987). In dasyurids the luteal phase occupies around 60% of the estrous cycle and following parturition, subsequent follicular activity is suppressed by lactation. This contrasts with the macropod pattern, where the luteal phase occupies ~90% of the estrous cycle and follicular growth is not suppressed, permitting post-partum estrus and ovulation to occur. Macropods also differ in that lactation prevents activity of the newly formed corpus luteum, resulting in a period of embryonic diapause. This discovery stimulated the onset of endocrine studies of macropods in the 1960s (Tyndale-Biscoe and Renfree, 1987) – research that has contributed strongly to our considerable understanding of their reproductive biology today. Unfortunately, limited attention has been directed toward detailed studies of the hormonal control of reproduction in many other marsupials, despite their diversity of reproductive strategies.

For the majority of marsupials, estrus and ovulation occur spontaneously, with the main hormones estradiol and progesterone acting on the female reproductive system in a manner analogous to that described for eutherian mammals (Tyndale-Biscoe and Renfree, 1987; Hinds *et al.*, 1996). Information is available on the reproductive physiology and breeding patterns for a range of non-macropod species, including the carnivorous dasyurids (Tyndale- Biscoe and Renfree, 1987; Tyndale-Biscoe, 2005), but fundamental knowledge of reproductive and endocrine parameters such as the temporal pattern of hormones and characteristics of the ovarian cycle is lacking. Dasyurids are characterised by a range of different reproductive strategies from semelparity to seasonal polyestry (Lee *et al.*, 1982; Krajewski *et al.*, 2000) but endocrine data is only available for several species (Fletcher, 1985; Hinds, 1989; Hinds and Selwood, 1990; Millis *et al.*, 1999). Our understanding of reproduction in the two largest dasyurids - the Tasmanian devil (*Sarcophilus harrisii*) and spotted-tailed quoll (*Dasyurus maculatus*) - remains relatively rudimentary, and even the most basic information such as timing of breeding,

estrous pattern and gestation length (reviews by Lee *et al.*, 1982; McAllan, 2003) is either assumed or inferred.

The Tasmanian devil is the world's largest extant dasyurid, reaching a bodymass of up to 12 kg (Strahan, 2005). Although the devil was formerly found on mainland Australia ~500 years ago (Jones *et al.*, 2003), wild populations are now confined to the island of Tasmania where, until recently, they were considered a common species of secure status. The outbreak of an apparently contagious and fatal facial tumor disease (Pearce and Swift, 2006) is currently having a devastating impact on wild devil populations, and the species is now listed as Threatened (Hawkins *et al.*, 2006). Despite devils being well represented in zoos for many decades, captive breeding remains inconsistent and limited, highlighting the need for a greater understanding of reproduction in this species (Carnio, 1993; Jackson, 2003). Detailed information of the devil's reproductive biology is now essential to implementing effective conservation programs, and is central to developing a fuller understanding of their natural life history and ecology.

Information is available on the basic reproductive biology (Guiler, 1970, 1971; Hughes, 1982), anatomy (Flynn, 1910, 1911; Pearson and De Bavay, 1953), oogenesis, spermatogenesis and embryology of the Tasmanian devil (O'Donoghue, 1912; Flynn, 1939; Hughes, 1982). There has, however, been no published research on their reproductive endocrinology, leaving important gaps in our fundamental understanding of this species. Most studies of the reproductive hormones during the estrous cycle of marsupials have relied on measuring concentrations of progesterone and/or estradiol in plasma; and have been conducted for a variety of species including macropods (Cake *et al.*, 1980; Walker and Gemmell, 1984; Hinds and Smith, 1992; Jones and Rose, 1992; Rose *et al.*, 1999), possums and opossums (Shorey and Hughes, 1973; Harder and Fleming, 1981; Curlewis *et al.*, 1985; Perret and Atramentowicz,1989; Hinds *et al.*, 1992; Hinds and Smith, 1992), the koala (Johnston *et al.*, 2000) and several dasyurids (*Dasyuroides byrnei* Fletcher, 1985; *Dasyurus viverrinus* Hinds, 1989; *Antechinus stuartii* Hinds, 1990; *Phascogale tapoatafa* Millis *et al.*, 1999). More recently, fecal steroid monitoring has begun to be applied to monitor female reproductive cycles in marsupials (Stead-Richardson *et al.*, 2001; Paris *et al.*, 2002; Bradshaw *et al.*, 2004; Woodd *et al.*, 2006). Fecal steroid monitoring of reproductive and stress hormones

confers obvious advantages over the restraint and handling associated with traditional blood collection, and this non-invasive method is particularly useful for more frequent monitoring, allowing for more thorough longitudinal assessment of endocrine cycles (reviews in: Lasley and Kirkpatrick, 1991; Schwarzenberger *et al.*,1996).

Knowledge of reproductive biology has been highlighted as being an essential component of conservation programs for Australian marsupials and is identified as having the greatest research priority (Temple-Smith, 2003). The main aim of this study was to provide the first detailed hormonal analysis of the ovarian cycle in a large dasyurid. A secondary aim was to evaluate the application of fecal steroid monitoring (total estrogens and progestagens) for the species as a basis for future applications of such techniques to in situ conservation and captive breeding. In a companion paper (Hesterman *et al.*, 2008b) (see Chapter 3), we describe the application of these techniques for monitoring ovarian cycles in the closely related spotted-tailed quoll.

2.3 Materials and Methods

2.3.1 Study animals and husbandry

Samples were collected from 14 captive female Tasmanian devils (0.5 - 6 years old) housed at Trowunna Wildlife Park (TWP Mole Creek, TAS) during 2001. Devils were provided with a natural diet consisting of kangaroo or wallaby meat and occasionally possum, rabbit, wombat or other native mammals/birds sourced from professional hunters or obtained as roadkill. Meat was provided complete with fur, bones and associated offal (except intestines), and animals were occasionally fed entire carcasses. Additional enrichment food items provided less often included commercially available brands of dog and cat biscuits, and whole raw carrots or apples. Water was available *ad libitum*.

Devils were housed outdoors, in naturalistic enclosures with access to climbing structures, native plants and other natural materials. Dens or nest boxes were available for shelter, with a choice of retreats that met or exceeded the number of animals per enclosure. Grouping of the study animals varied during the year, with devils generally kept in mixed sex or same sex groups and separated for breeding requirements or experimental purposes. Introductions and pairing with males were permitted for

selected females during the breeding season, in accordance with experimental design (see below).

2.3.2 Experimental design

To assess and compare the estrous and pregnant cycles, 11 of the 12 adult females (≥2 years) were assigned to different groups:

Group A: Females permitted full access to males during estrus (mated; n = 6); and

Group B: Females with restricted access to males during estrus (nonmated; n = 5).

The first group of animals were housed together in a female-only group for at least 1 month prior to the onset of breeding, and removed for pairing with males at the onset of behavioral and physical cues used by park management to detect estrus. Females removed for mating were placed into a specifically designed enclosure, with free access to a choice of two adult males housed in adjacent wire pens. Copulation was confirmed by behavioral observation (observer presence/video monitoring within dens), or detection of sperm in the vaginal smear. Following mating and/or lack of interest or increased aggression toward males, females were removed to individual enclosures.

The second group of animals was also housed together in a female-only group prior to the onset of breeding season. Between February and March they were periodically exposed to an adult male: three times each week, a male was placed into a cage (1.5 x1.0x 0.9 m) within the females' enclosure for 30 – 60 min while behavior was recorded for a concurrent study. To compare mated and non-mated estrous cycles in the same individuals, Group B females were housed with unrestricted access to a male throughout April–May.

2.3.3 Sample and data collection

Plasma collection

Blood samples were collected during routine handling of animals to obtain supplementary data on reproductive status (see below). Devils were captured by hand or use of a large net. They were restrained unanaesthetised in a sack during sample collection and examination. Blood was collected within five minutes of capture.

A peripheral ear vein was pricked with a disposable Stat-Let© lancet and 75–150 µl blood was collected via a heparinised capillary tube. Samples were usually taken between 0730 and 0930 h or 1500 and 1700 h except when individuals were being captured for other husbandry purposes, when blood was collected opportunistically. Samples were kept at 4 °C until centrifuged later that day, and the plasma was recovered and stored frozen (-20 °C) until radioimmunoassay.

Blood was collected from adult devils at intervals of ~5 days during late January–May, with frequency increasing to every 2 – 3 days from the onset of estrus. For females that were mated, sampling was discontinued 2 weeks post-copulation to minimize any potential stress that might impact on successful rearing of young. Sampling resumed if subsequent pouch checks confirmed no young were present. Subadult devils (1 year olds; n = 2) were sampled at ~10-day intervals from late February–May.

Fecal collection

Fecal samples were collected between November 2000 and December 2001. To ensure the identity of individual samples when animals were housed together, small colored plastic beads (3 mm diameter) were mixed into a mincemeat ball and fed to the study animals the day before collection. Color coding was consistent for each animal and up to six different individuals could be identified within a single enclosure. Frequency of collection varied depending on the time of year and breeding status of individual animals. Immediately prior to and during the mating season (February – May), samples were obtained up to three times per week, whereas during the rest of the year samples were collected on a weekly or fortnightly basis.

Entire feces were usually collected during morning servicing (0730 – 0900 h) or opportunistically when freshly voided throughout the day. When several scats were available from the same individual, the apparently freshest sample was selected. Samples were placed in zip-lock plastic bags and stored at -20 °C for later processing. Frozen samples were freeze-dried (Dynavac FD16, Dynavac High Vacuum Pty. Ltd., Victoria, Australia) and screened through 1 mm^2 plastic mesh to remove fur, bones and other fibrous or undigested matter; screened feces were stored refrozen in ziplock bags. These samples were shipped frozen to Vienna, Austria and kept at -20 °C until analysis.

Additional data and sampling

Vaginal (urogenital) smears were collected from all females paired with males, and also from three non-mated individuals. Smears were obtained from the posterior vaginal sinus by introduction of a small cotton swab through an appropriately sized glass speculum (70 mm length x 5 mm ø).Smears were air-dried, fixed and then stained with acid fuchsin and toluidine blue following methods outlined in Dix and Billings (1969). Stained smears were examined for percentage of intermediate (IE) and superficial/cornified epithelials (SE), leucocytes and presence of spermatozoa. Pouches were monitored for condition/presence of young.

Plasma progesterone analyses

Plasma progesterone was measured by radioimmunoassay (RIA). 40 µl duplicates of plasma were extracted in 1 ml iso-octane (AnalaR grade APS Ajax Finechem, NSW, Australia) by brief vortexing followed by incubation at room temperature for 2 h. Samples were frozen at -20°C for 30 min to allow phase separation. The solvent was evaporated, the extract redissolved in assay buffer and used for radioimmunoassay. Recovery varied between samples, so extraction efficiency was assessed for individual samples. Average recovery of radioactive progesterone was 85% (± 0.5 S.E.; range 65 - 95%).

For the assay 50 µl [3H] progesterone ([1,2,6,7] (TRK413 Amersham Pharmacia Biotech, UK)) containing ~12 000 cpm was added to tubes containing sample extracts and progesterone standards (range 3.12 – 200 pg/50 µl; Sigma-Aldrich Pty, Ltd, Missouri, U.S.A.) and evaporated under air. 100 µl of antiserum (P11 – 192, Endocrine Sciences, California, USA) diluted 1:125 000 in phosphate buffer (PBSG 0.05 M pH 7.4; 0.1% gelatin) was added to dried extracts and standards. Cross-reactivities of this antiserum with other steroids are: 4-pregnen-20β-ol-3-one (1.3%), 4-pregnen-20α-ol-3-one (0.8%), 17α-hydroxyprogesterone (0.6%), deoxycorticosterone (3.3%), corticosterone (0.6%), 11-desoxycortisol (0.4%) and all others (<0.1%). The tubes were vortexed briefly and incubated at 4°C overnight. Unbound steroid was separated by addition of 500 µl dextran-coated charcoal (0.5g/l charcoal (Sigma-Aldrich Pty, Ltd, Missouri, U.S.A.), 0.05g/L dextran (Dextran T70 Amersham Pharmacia, Buckinghamshire, England) in phosphate buffer and incubation on ice for 15 min, then centrifuging for 15 min (1500 g at 4°C). 300 µl of supernatant was counted in 2.5 ml

scintillation fluid (Ecolite, MP Biomedicals, Inc. California, USA) for 5 min in a Beckman Coulter Counter LS 5801.

Assay sensitivity was 3 pg/tube (0.09 ng/ml). Serial dilutions of devil plasma ran parallel to the progesterone standard curve. Recoveries of added steroid were determined by spiking pooled plasma for each species with progesterone (0.5, 1.0, 2.0, 4.0 ng/ml) which yielded a mean recovery within 10% of expected values. Multiple aliquots from a pool of female devil plasma were extracted to measure intra-assay variability, whereas commercially available controls (Diagnostic Products Corporation, California, USA) were used to monitor inter-assay variation. Intra- and inter-assay coefficients of variation were 9.5% (n = 9) and 14.8% (n = 9), respectively. All samples from each individual were included in a single assay.

Fecal sample processing and enzyme-immunoassay of steroids

Lyophilized fecal samples were mixed with distilled water (0.1 g faces in 0.9 ml water), and then extracted in methanol and diethyl ether, as described in Schwarzenberger *et al.* (2000). After adding 4.5 ml of methanol, the fecal-water-methanol mixture was vortexed (30 min) and centrifuged. Thereafter, 1.0 ml of the methanol supernatant was recovered into a separate vial and 0.5 ml of a 5% $NaHCO_3$ in water solution were added, and then extracted with 3.0 ml of diethyl ether. The ether phase was collected, evaporated to dryness and the extract residue redissolved in assay buffer. After appropriate dilution (1:100 to 1:1000 depending on steroid concentration) immuno-reactive progesterone and estrogen metabolites were assayed using previously established group-specific enzyme-immunoassays (EIA) (Schwarzenberger et al 1997). Samples were analyzed for 20α-OH-pregnanes (antibody: 5ß-pregnane-3α-20α-diol 3HS:BSA; trivial name pregnanediol), 20-oxo-pregnanes (antibody: 5α-pregnane-3ß-ol-20-one 3HS:BSA), and total estrogens (antibody: oestradiol-17ß-OH 17-HS:BSA). Preliminary testing showed that assay of 20α-OH-pregnanes (pregnanediol PgD) was most appropriate, with concentrations being excreted in consistently higher levels than 20-oxo-pregnanes throughout all stages of the estrous cycle. EIAs were validated by demonstrating parallelism between standard curves and serial dilutions of the fecal extracts and by showing that fecal values followed the same trend as the values obtained with the

plasma progesterone assay. The intra- and inter-assay coefficients of variation for the assays tested were below 10% and 15% respectively.

Terminology

Various terminology has been used to describe non-conceptive cycles in marsupials - whether not mated, mated or hormonally/mechanically stimulated - as either non-pregnant, pseudopregnant or failed pregnant. To avoid ambiguity, the term non-mated is used here to distinguish between non-pregnant and pregnant cycles. Apart from a single individual for which neither mating nor birth could be confirmed, all females paired with males gave birth: information on that individual was excluded from comparative analyses of pregnant and non-mated cycles.

2.3.4 Interpretation of hormone data

Stages of the estrous cycle were defined as the follicular phase (FP),luteal phase (LP), anestrous and inter-estrus (period between beginning of FP to onset of next FP). The FP has a secondary stage where ovulation is presumed to occur. This distinct stage reliably follows the hormone surge during the FP, and is distinguished by a trough/return to baseline values for fecal estrogen (and also plasma progesterone concentrations) prior to onset of the LP. Where collection frequency was ≥ 7 days, the durations of successive stages of the cycle were calculated by counting the days elapsed between the two samples, halving the result and adding it to the phase either side.

Onset of the estrous cycle could be determined without monitoring plasma estrogens because plasma progesterone concentrations characteristically rose at pro-estrous. Baseline values for plasma progesterone were generated by averaging values obtained from mature females during anestrous. Increases above the group baseline + one standard deviation (SD) (*i.e.* >0.48 ng/ml) that were maintained for at least 5 days were considered indicative of onset of the FP. Confirmation of the LP was readily identified when plasma progesterone concentrations increased two SDs above baseline levels (*i.e.* >0.64 ng/ml).

Group baseline values were calculated by averaging fecal steroid concentrations in adults during the non-breeding season (n = 10 animals, 72 samples). The onset of the estrous cycle was defined when fecal estrogen concentrations rose above the group baseline + one SD (*i.e.* > 25.34 ng/g), and remained elevated for at least 1 week. Onset

of the LP was marked by rising and sustained concentrations of pregnanediol which exceeded the group mean (*i.e.* >1036 ng/g). The end of the LP was identified as the time when fecal pregnanes dropped below the mean with subsequent samples remaining low for at least 2 weeks; or, for pregnant females was terminated by birth, indicated by presence of young in pouch (PY). Where date of birth was uncertain (in two cases only), the age of the PY was estimated from measurements and backdating from established growth curves for the species (Phillips and Jackson, 2003; Appendix C).

2.3.5 Comparison between plasma and feces

To allow comparison between the pattern of excretion between plasma progesterone and fecal progestagens, temporal alignment of samples was necessary. Fecal steroids mimic the pattern of circulating hormone levels in plasma, but incur a lag time due to their passage through the gut (Schwarzenberger *et al.*, 1996), roughly equivalent to passage of digesta (Lasley and Kirkpatrick, 1991; Schwarzenberger *et al.*, 1996). Accordingly, fecal samples were displaced from the plasma results by 24 h, based on the time of appearance of indicators (small, colored plastic beads) fed to individually identify scats.

2.3.6 Statistical analyses

All data are presented as means ± SE, except where indicated otherwise. Student's unpaired t test was used for comparison of estrous characteristics, and duration between pregnant cycles and mated or non-mated non-parturient cycles. Analyses of variance (ANOVA) were used to detect temporal changes in hormone profiles, and to test for significant differences in hormone concentrations between experimental groups. For comparison of the profiles for plasma progesterone and its fecal metabolites, regression was performed on data that were log-transformed to meet the assumptions of ANOVA. Statistical analyses were performed using SPSS (SPSS Inc. 1998, Chicago IL), Version 13 package.

2.4 Results

Twelve of the 13 female devils monitored during the study period underwent estrous cycles, and seven pregnancies were recorded. None of the devils that mated and produced young returned to estrus. Five of the eight individuals that were not mated at first estrus underwent a second cycle in the breeding season, which lasted approximately six months from the end of January until late June.

2.4.1 Estrous cycle characteristics

Characteristics of the estrus cycle are summarized in Table 1. The mean duration of the estrous cycle was approximately 32 days whether assessed by plasma or fecal hormones. There was no significant difference between the length of the FP (~14 days) in non-mated and mated animals ($t_{(11)} = 1.09$, $P = 0.30$). Analyses of plasma progesterone data indicated no difference in the duration of the nonmated FP whether females were physically exposed to males (11.2 ± 1.9 days; n = 5) or not (11.0 ± 1.0 days; n = 2) at estrus. Duration of the FP based on fecal estrogen concentrations suggested a difference between those two groups (male exposure 16.4 ± 1.9 days vs. no male exposure 9.25 ± 1.3 days), but sample sizes were too limited for statistical analysis. The inter-estrus period lasted between two to three months (Table 1).

2.4.2 Plasma progesterone

Plasma progesterone concentrations for adult female devils sampled during the breeding season averaged 1.64 ± 0.3 ng/ml; with the lowest values from an immature (first year) animal that did not cycle, approximating basal levels (<0.5 ng/ml) in all other study animals. The pattern of plasma progesterone concentrations was similar during estrous and pregnancy (Fig. 1a and b). Progesterone concentrations were initially low, but a small well-defined pulse (1.33 ± 0.2 ng/ml) occurred approximately two weeks prior to onset of the LP. Pro-estrous concentrations increased up to a maximum of 3.39 ng/ml about 10 days prior to luteal onset, and were associated with presence of an estrus vaginal smear (>95% mature epithelial cells) (Fig. 2), and copulation for females paired with males. Following the pro-estrous pulse, plasma progesterone concentrations returned to baseline for 5.0 ± 0.5 days (range 3 – 9 days), but during the LP mean concentrations reached 5.28 ± 0.8 ng/ml. Progesterone concentrations peaked approximately two weeks after the luteal onset (max 22.82 ng/ml), but dropped rapidly soon afterward. Copulations were recorded over varied intervals, from a single day to recurrent bouts over a period of up to 8 days in length (3.4 ± 0.9 days). Matings occurred immediately prior to, during and after the pro-estrous peak in progesterone. Females gave birth 20 – 31 days from first copulation. Animals that were not mated returned to estrus by day 65 (range 46–89) (*e.g.* Fig. 2), and two of the three females paired with a male in their second cycle produced young.

Table 1

Characteristics of the estrous cycle in the Tasmanian devil as assessed by changes in fecal (estrogens and progestagens) and plasma (progesterone) sex steroid concentrations.

	FECES		PLASMA	
	Mean length ± S.E. (days)	No. cycles (individuals)	Mean length ± S.E. (days)	No. cycles (individuals)
FOLLICULAR PHASE (FP)				
Non-mated	14.9 ± 1.5 (range 8 – 21)	8 (8)	11.3 ± 1.4 (range 9 – 17)	9 (7)
Mated	14. 9 ± 2.1 (range 8 – 23)	7 (7)	13. 7 ± 1.9 (range 9 – 17)	6 (6)
LUTEAL PHASE (LP)				
Non-mated	17.9 ± 0.3 (range 12 – 25)	8 (8)	20.6 ± 1.0 (range 15 – 29)	10 (6)
Pregnant	16. 7 ± 0.8 (range 14 – 21)	7 (7)	n/a	n/a
ESTROUS CYCLE (FP + LP)				
Non-mated	31.4 ± 1.8 (range 21- 40)	8 (8)	32.1 ± 1.4 (range 27 – 42)	10 (12)
Pregnant	32.1 ± 2.7 (range 24 – 39)	6 (6)		
INTER-ESTRUS (FP to FP)	80.3 ± 7.1 (range 64 - 102)	6 (6)	73.3 ± 7.3 (range 56 – 99)	5 (5)

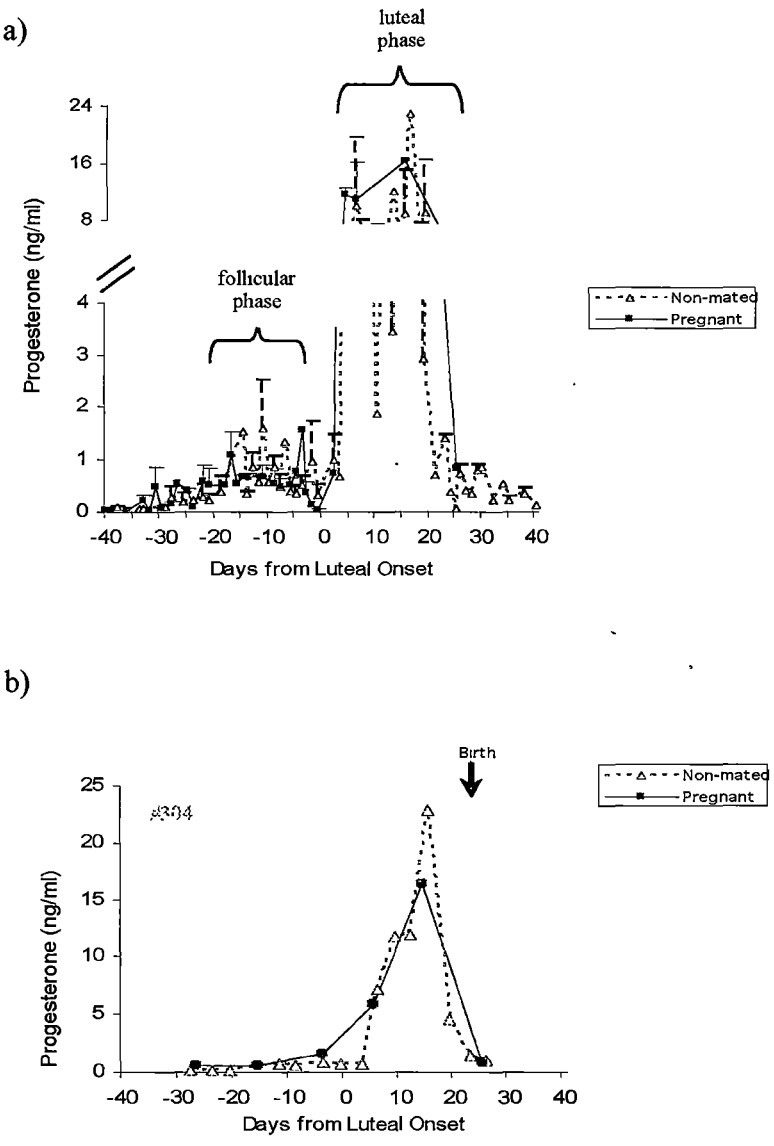

FIG. 1

a) Mean profile and b) an individual plasma progesterone (ng/ml) profile for non-mated (--Δ--) and pregnant (●) Tasmanian devils between days -40 to 40 from luteal onset (day 0). Note pro-estrous surge and change in scale of y-axis for plasma progesterone concentrations in 1a).

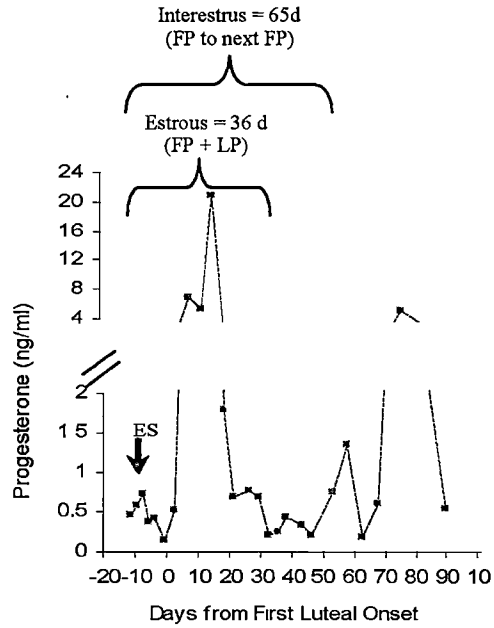

FIG. 2

Individual plasma progesterone profile (ng/ml) in a non-mated female Tasmanian devil (#201) sampled days - 20 to 100 from onset of first luteal phase. ES indicates collection of an estrus smear. Note change in scale of y-axis. Estrous and inter-estrus interval (duration between follicular phases (FP) shown.

2.4.3 Comparisons between fecal metabolites and plasma progesterone

Pregnanediol (PgD) was excreted in consistently higher concentrations than 20-oxo-pregnanes, and during the breeding season PgD concentrations were around 8-to 11-fold higher by comparison. The pattern of excretion was very similar for the two fecal metabolites (Fig. 3), and during the estrous cycle fecal progestagens and plasma progesterone concentrations showed a significant positive correlation ($P < 0.05$; mean slope: $y = 0.91 \pm 0.2$).

2.4.4 Fecal steroid profiles

There was a highly significant difference in mean concentrations of fecal PgD and total oestrogens during the breeding and non-breeding season (PgD non-breeding: 219.70 ± 29.16 ng/g, breeding: 1634.50 ± 191.56 ng/g ($t_{(215)} = -4.226$, $P < 0.001$; oestrogens non-breeding: 8.56 ± 2.59 ng/g, breeding: 23.84 ± 2.50 ng/g ($t_{(217)} = -3.367$, $P = 0.001$)). Fecal PgD and 20-oxo-pregnanes were significantly positively correlated ($y = 1.05x + 1.21$, $R^2 = 0.54$; $P < 0.01$).

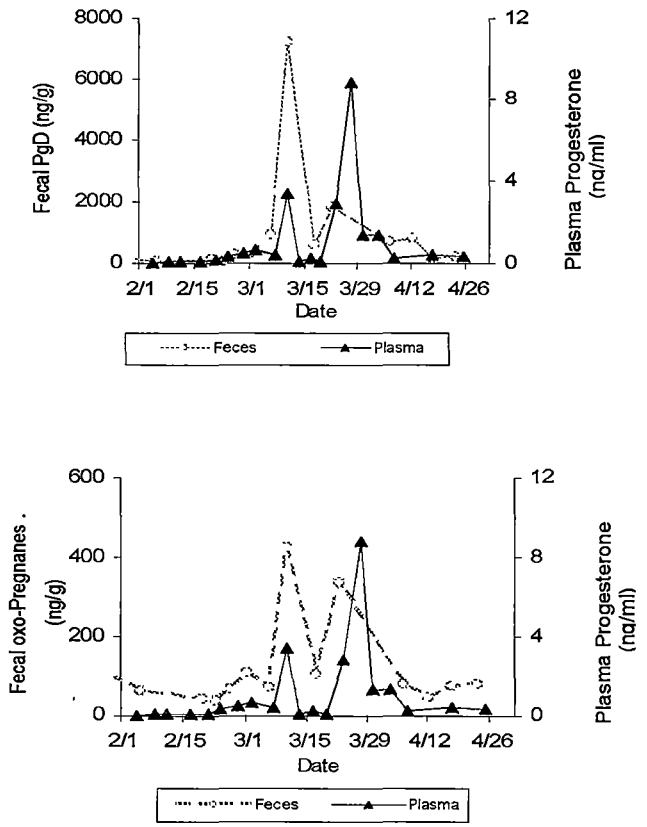

FIG. 3

Fecal progestagens (20-α-pregnanes and 20-oxo- pregnanes) (ng/g) and plasma progesterone (ng/ml) concentrations in a female Tasmanian devil. Fecal samples were displaced from plasma to correspond with approximate steroid lag time of one day (24hrs). Note biphasic pattern due to characteristic pro-estrous pulse in progesterone/progestagens during the follicular phase.

Non-mated estrous cycle

When aligned to days from luteal onset, PgD profiles for non-mated devils during the estrous cycle were strikingly similar (Fig. 4). There was a relatively minor, and brief pro-estrous increase in PgD concentrations up to 2 weeks prior to onset of the LP (<6500 ng/g) and a major increase in total estrogen levels. Immediately after luteal onset (day 0), PgD concentrations surged dramatically to reach a sharp peak within 2–5 days. Elevated PgD concentrations were sustained until around day 15–20, and then declined to near baseline measures (~200 ng/g). Most animals returned to estrus between days 47 and 68. To illustrate the pattern of fecal steroids during the non-mated estrous cycle

more clearly, individual profiles from two females are shown in Fig. 5. Elevated fecal estrogen concentrations occurred 1–2 weeks prior to onset of the LP, and timing of peaks related to occurrence of estrus smears (>95% cornified cells) (Fig. 5a). The pattern of estrogens during the LP was inconsistent between individuals. Five of the eight devils observed during non-mated cycles underwent a second cycle, including three females that were paired with males at their second estrus (*e.g.* Fig. 5b).

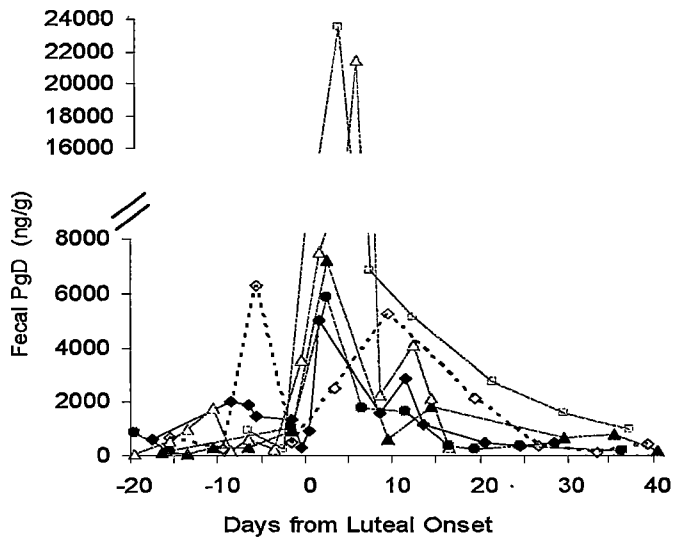

FIG. 4

Fecal pregnanediol (PgD) concentrations (ng/g) in six non-mated female Tasmanian Devils aligned to onset of luteal phase. Note break in y-axis.

Pregnant estrous cycle

Fecal hormone profiles for pregnant devils were aligned to day of final mating (day 0) (Fig. 6), due to the variation between individuals in the period between first and final copulation (1–7 days). The biphasic pattern of fecal PgD was similar to that observed during the non-mated cycle (Fig. 4). A brief surge of PgD occurred within five days after last mating, and the major peak occurred on day 18: levels returned to baseline by days 25–30 in most animals. Parturition occurred within a few days of the precipitous decline. Births were recorded on days 19, 23, 24, 26(2) and 27(2) from final mating.

Individual profiles for pregnant devils (*e.g.* Fig. 7) show that sustained increases in fecal estrogen concentrations occurred up to three weeks prior to mating; copulation occurred within ~3 days of the concurrent peak in fecal estrogens and PgD. During the LP fecal estrogen concentrations were low, compared to during the FP. Fecal PgD and total estrogen concentrations remained low (<400 and <20 ng/g, respectively) throughout lactation.

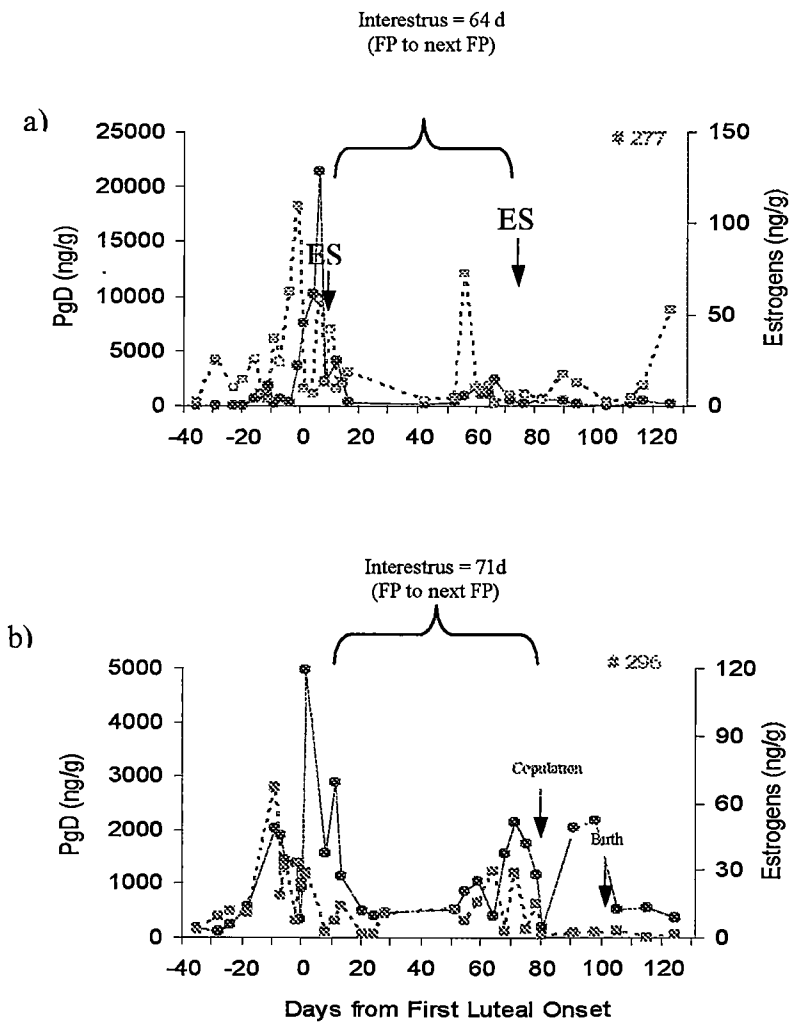

FIG. 5

Fecal pregnanediol (PgD) (•) and total estrogen (□) concentrations (ng/g) in two female Tasmanian devils between d -40 and 120 from first luteal onset (day 0). Female in a) not mated during either estrus; female in b) mated during second estrus; copulation and parturition indicated by arrows. ES indicates collection of an estrus smear. Inter-estrus interval shown in brackets for each individual; FP = follicular phase.

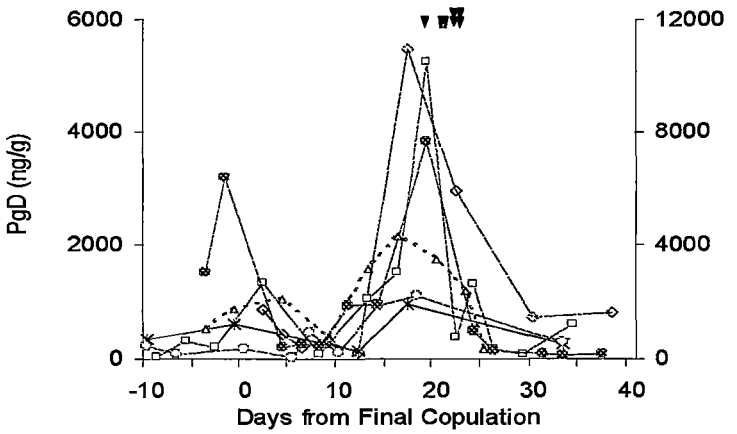

FIG. 6

Fecal pregnanediol (PgD) concentrations (ng/g) between days -10 to 40 days from final mating of that breeding season in six pregnant Tasmanian Devils. Note second y axis also displays fecal PgD (ng/g), due to elevated concentrations in one individual (●). Births (▼) occurred from days 19 to 27; stacked triangles depict the number of animals giving birth on that day.

2.4.5 Comparison of the non-mated and pregnant estrous cycle

There were no significant differences between mean concentrations of fecal steroids in pregnant and non-mated devils ((total estrogens = 16.74 ± 2.7 ng/g versus 22.72 ± 3.0 ng/g, respectively; $t_{(134)} = -1.42$, $P = 0.16$) (PgD = 1045.0 ± 274 ng/g versus 1217.7 ± 175 ng/g, respectively; $t_{(103)} = 1.00$, $P = 0.32$)) (Fig. 8). ANOVA showed a significant difference in fecal PgD and estrogen concentrations over time for both groups (PgD mated: $F = 2.641$, $P = 0.02$, non-mated: $F_{(7,42)} = 2.777$, $P = 0.02$; estrogens mated: $F_{(7,49)} = 2.926$, $P = 0.01$, non-mated: $F_{(7,42)} = 3.058$, $P = 0.01$). Fecal estrogens were high in both groups prior to luteal onset (days -15 to -1), but between days 0 to 5 there was a significant difference in estrogen levels between the two groups ($P < 0.05$): estrogens declined briefly in pregnant females but increased significantly in non-mated females (Fig. 8a) Between days 6–10 an inverse effect was observed, with estrogens rising in pregnant devils but dropping significantly ($P < 0\ 05$) in non-mated animals. In both groups, fecal estrogens returned to baseline between days 21–25.

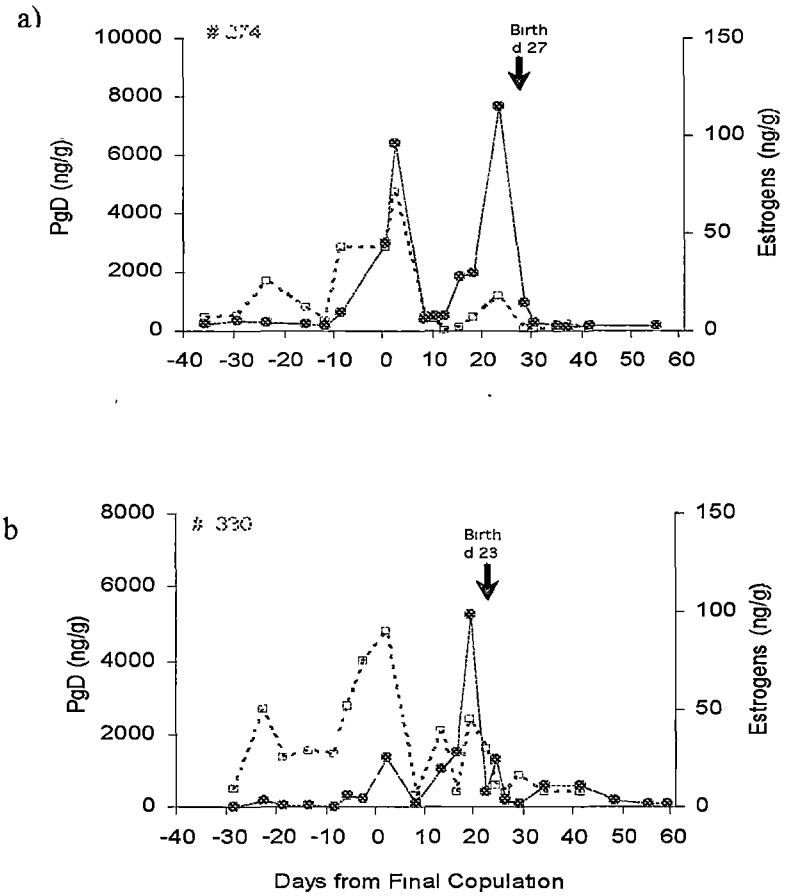

FIG. 7.

Fecal pregnanediol (PgD) (•) and total estrogen (□) concentrations (ng/g) in two pregnant Tasmanian devils. Profiles are aligned from day of final copulation during that breeding season, with parturition indicated by arrows.

Fecal PgD concentrations were lowest between days -15 to -11 in both groups, but a major increase was observed for non-mated females only between days -10 to -6 (Fig. 8b). Mean PgD levels remained unchanged in females paired with males prior to luteal onset, and were similar between the two groups from days -5 to -1. A significant difference in mean PgD concentrations was observed between the groups on days 0–5 ($t_{(17)} = 2.72$, $P = 0.02$), with non-mated devils having a rapid increase in PgD ($P < 0.05$), which steadily declined throughout the remainder of the LP (days 6–25). In contrast, for pregnant devils PgD levels increased slowly from luteal onset (day 0) and peaked between days 11–15. Births occurred during days 14– 22, during the subsequent progestagen decline.

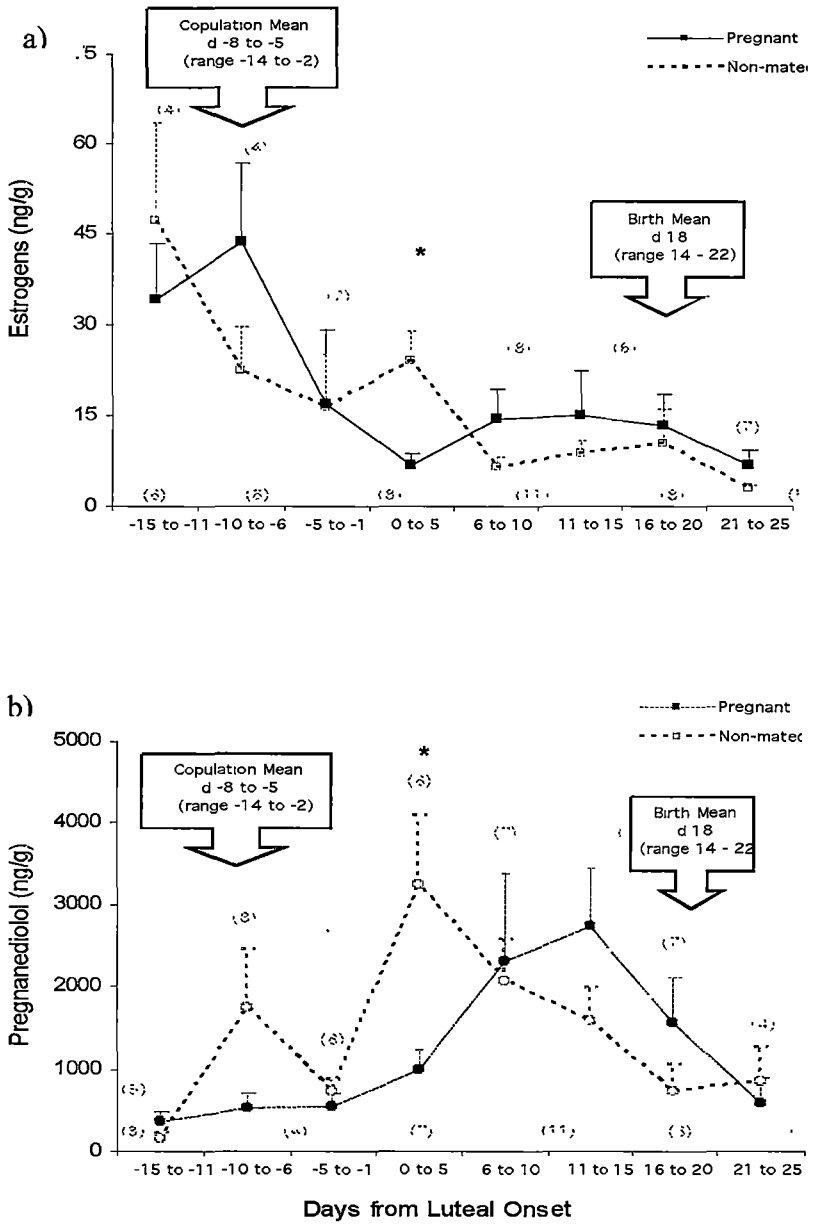

FIG. 8

Grouped mean profiles comparing a) fecal estrogens and b) pregnanediol (PgD) concentrations (ng/g) in non-mated and pregnant Tasmanian devils between days -15 to 25 from luteal onset. Mean copulation interval (first to final mating) and range indicated by arrows; mean day of birth (from luteal onset) and range also presented. Sample number indicated in brackets nearest to respective mean. Asterix * indicates a significant difference between treatment groups ($P < 0.05$).

2.5 Discussion

2.5.1 Characteristics of the estrous cycle

Through endocrine monitoring we established that the duration of the estrous cycle in the Tasmanian devil is around 32 days. Studies of other dasyurids based on plasma progesterone concentrations have confirmed estrous as ranging from ~37 days in the eastern quoll (Hinds, 1989) to up to ~60 days in the kowari (Fletcher, 1985). Longitudinal profiling confirmed that the devil is a spontaneous ovulator, and is seasonally polyestrous - similar to most other marsupials (Tyndale-Biscoe and Renfree, 1987; Hinds *et al.*, 1996). Due to the lengthy period of lactation in Tasmanian devils (Russell, 1982), females that conceive at first estrus and retain those young do not cycle again during the breeding season. Non-mated devils did not return to estrus immediately following the end of one cycle, and the inter-estrus interval (onset of FP to next FP) was 2–3 months, similar to that reported for kowari (Fletcher, 1985). Longitudinal profiles showed a maximum of two estrous cycles in one year.

2.5.2 Pattern of the estrous cycle

The follicular phase

The characteristic biphasic pattern of plasma progesterone concentrations during the estrous cycle has previously been reported for the eastern quoll (Hinds, 1989) and the kowari (Fletcher, 1985), but not in non-dasyurid species (Shorey and Hughes, 1973; Curlewis *et al.*, 1985; Gemmell *et al.*, 1987; Johnston *et al.*, 2000; Woodd *et al.*, 2006). The duration of the pro-estrous rise in plasma progesterone in devils was 8–10 days, similar to in the kowari (Fletcher, 1985) and eastern quoll (Hinds, 1989). The source of progesterone at this time is proposed to be developing or luteinized ovarian follicles rather than corpora lutea (CL) (Fletcher, 1985; Hinds, 1989), because the increase precedes the first estrus (Fletcher, 1985).

Multiple, brief peaks of fecal estrogens were sometimes observed during estrus, similar to the wave-like pattern of fecal estradiol-17b reported for chuditch (*Dasyurus geoffroii*) (Stead-Richardson *et al.*, 2001). Increases in fecal estrogens often preceded the onset of estrus, but when they were accompanied by a pro-estrous rise in fecal progestagens, they

coincided with fully cornified vaginal smears and copulation. As reported for other dasyurids (Fletcher, 1985; Hinds, 1989; Stead-Richardson *et al.*, 2001), mating occurred immediately prior to, during and/or following these peaks in plasma or fecal hormones. Copulations occurred over a typical 1–2 days time-frame (Tyndale-Biscoe and Renfree, 1987), but in several individuals mating was prolonged for up to 8 days, in accordance with previous reports for the species (Smith, 1993). A relatively protracted estrus period has also been observed in some other dasyurids, including Antechinus (Tyndale-Biscoe and Renfree, 1987) and the chuditch (Stead-Richardson *et al.*,2001), and is thought to be related to sperm storage and delayed ovulation (Taggart *et al.*, 2003).

After the pro-estrous peak, there was a 3 to 9 day nadir in plasma progesterone and fecal progestagen concentrations. This temporary decline in progesterone is also evident in the eastern quoll (Hinds, 1989) and kowari (Fletcher, 1985), and is presumed to be the period during which ovulation occurs (Selwood, 1982; Fletcher, 1985; Hinds, 1989). Most marsupials ovulate within 1–2 days of estrus, but in dasyurids the interval is longer (Tyndale-Biscoe and Renfree, 1987; Taggart *et al.*, 2003). In the Tasmanian devil, mean duration of this 'ovulatory interval' was approximately 7 days. Ovulation is thought to take place around 4–6 days post-estrus in the eastern quoll and kowari (Hill and O'Donoghue, 1913; Fletcher, 1985), and up to 10 days post-estrus in the brown antechinus (A. stuartii) (Selwood, 1980). Considerable variation in time to ovulation occurs in eastern quoll (Hinds, 1989), which could account for the extended interval we observed.

The luteal phase

The pattern of plasma progesterone concentrations during the luteal phase (LP) in Tasmanian devils resembled that described for other non-macropodid marsupials (Tyndale-Biscoe and Renfree, 1987; Hinds, 1989; Hinds and Selwood,1990; Bradshaw and Bradshaw, 1992; Millis *et al.*,1999; Johnston *et al.*, 2000). The primary source of progesterone is the autonomous CL (Tyndale-Biscoe and Renfree,1987; Hinds, 1990; Gemmell, 1995); and in the eastern quoll (Hinds, 1989) and brown antechinus (Hinds and Selwood, 1990) the major sustained increase in plasma progesterone during the LP mirrors CL development. For devils, births occurred within days of a precipitous decline

in progesterone/progestagen concentrations, probably in association with the demise and involution of the CL (Hinds and Selwood, 1990).

Temporary elevations in estrogens during the LP have also been noted in the chuditch, squirrel glider (*Petaurus norfolcensis*) (faecal: Stead-Richardson *et al.*, 2001; Woodd *et al.*, 2006) and American opossum (*Didelphis virginiana*) (plasma: Harder and Fleming, 1981). This occurrence probably reflects incomplete suppression of ovarian activity, with the follicles failing to mature (Fleming and Harder, 1983).

2.5.3 Duration of pregnancy

The period over which matings occurred varied widely (1 – 8 days) indicating that, as for other dasyurids, ovulation did not occur at a fixed time in relation to estrus. For this reason, the interval from mating to birth (21 – 62 days) is frequently cited as the gestation period for dasyurids (Tyndale-Biscoe and Renfree, 1987). Previous estimates for devils vary from 19 days (Slater, 1993) to 28 – 31 days (Guiler, 1970; Guiler, 1971), and we determined a comparative figure of ~24 days (24.6 ± 1.1 days; range 19– 27 days) through calculating time from final mating to parturition. Selwood and Woolley (1991) note, however, that the day of copulation is not a useful indicator of onset of embryonic development. In addition to the variable interval from mating to ovulation, the period of sperm storage may also be variable (Taggart *et al.*, 2003). Therefore, the interval from luteal onset to birth provides a more realistic and accurate gestation length of 17.9 ± 1.0 days (range 14 – 22 days) for the Tasmanian devil.

2.5.4 Comparison of the mated and non-mated estrous cycle

There was no difference in the mean length of the FP (which at ~15 days is similar to other dasyurids: Tyndale-Biscoe and Renfree, 1987) between mated and non-mated devils. As in most marsupials (Fletcher, 1985; Tyndale-Biscoe and Renfree, 1987; Hinds, 1989; Hinds, 1990; Hinds and Selwood, 1990), there were no differences in the mean duration of the LP or in the magnitude/duration of plasma progesterone profiles between the mated and non-mated estrous cycle. Documenting maternal recognition of pregnancy is difficult in marsupials, not only because there are usually no obvious differences in hormone profiles, but because the conceptus is encapsulated in an

extracellular shell membrane prior to implantation (Cruz *et al.*, 2001). However, through monitoring fecal sex steroids, our study revealed intriguing differences in the pattern of estrogen and progestagen excretion between the mated and the non-mated estrous cycle in devils. The signature pattern of sex steroids varied during periods associated (in pregnant animals) with blastocyst expansion and implantation (Hinds and Selwood, 1990): these results suggest maternal endocrine recognition of pregnancy may occur in this species.

This study has provided fundamental information on the reproductive endocrinology of *S. harrisii*, the largest species of dasyurid, and provides a detailed description of the estrous cycle. It has also demonstrated the effectiveness of faecal steroid analyses for monitoring the ovarian cycle in this now-endangered marsupial. This research represents the first analysis of plasma progesterone concentrations in the devil, and the first longitudinal assessment of fecal sex steroids (estrogens and progestagens) in a dasyurid. Correlations between patterns of plasma and fecal sex steroid concentrations and physiological events including copulation, pregnancy and birth confirm the validity of fecal steroid measurements as an alternative, non-invasive application for monitoring female reproduction in this species.

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CHAPTER 3

PLASMA AND FECAL STEROID MONITORING OF OVARIAN CYCLES IN THE SPOTTED-TAILED QUOLL (*DASYURUS MACULATUS*)

3.1 Abstract

Dasyurids exhibit a range of breeding patterns from semelparity through to an aseasonally polyestrous strategy, but detailed information on the reproductive endocrinology of many species is unavailable. This study aimed to extend our comparative understanding by characterizing the ovarian cycle of the spotted-tailed quoll (*Dasyurus maculatus*) through measurement of plasma progesterone, and also to investigate fecal sex steroid monitoring as an alternative, non-invasive technique. Longitudinal profiles revealed a biphasic pattern of plasma progesterone, with a significant pro-estrous pulse (0.97 ± 0.3 ng/ml) up to several weeks prior to onset of the luteal phase (LP). This pro-estrous period was associated with a predominantly cornified vaginal smear, onset of estrus behaviors and copulation. Mean luteal values for plasma progesterone were several fold higher (2.18 ± 1.10 ng/ml) than during the follicular phase (FP) (0.75 ± 0.02 ng/ ml), and were sustained for approximately one month. Fecal progestagens and plasma progesterone were significantly positively associated during the estrous cycle. During the breeding period average concentrations of fecal total estrogens and pregnanediol (PgD) were significantly elevated. Ovarian activity during the FP was marked by increases in fecal estrogens, and rises in PgD which were sustained during the LP. In non-mated females the mean duration of the FP was significantly extended, being approximately twice as long (19.4 ± 4.0 d) as for mated females (8.3 ± 1.9 d) indicating coitus has some role in timing of ovulation in this species. This study has provided important new information on the reproductive biology of the female spotted-tailed quoll, and further demonstrated the usefulness of non-invasive endocrine techniques for monitoring ovarian cycles in marsupials.

3.2 Introduction

The Dasyuridae are a taxonomically diverse family, represented by more than fifty extant species, most of which live in Australia (Krajewski and Westerman, 2003). Dasyurids have evolved a wide range of life history strategies characterized at one extreme by a strictly regulated monoestrous cycle in females and the abrupt post-mating death finale of males (*e.g. Antechinus*) through to aseasonally polyestrous species which produce several litters each year (Lee *et al.*, 1982;Krajewski *et al.*, 2000;McAllan, 2003). Despite considerable focus on the reproductive biology of the semelparous Antechinus (Woolley, 1966; Selwood, 1980, 1985; Taggart and Temple-Smith, 1991), and detailed studies of several other dasyurids (Hill and O'Donoghue, 1913; Fletcher, 1985; Hinds, 1989; Selwood and Woolley, 1991) data on many species is still unavailable (Krajewski *et al.*, 2000). There has been little additional research on the reproductive endocrinology of dasyurids in nearly 20 years.

Large marsupial carnivores have undergone major anthropogenic declines, and are predisposed to extinction events by a number of factors including naturally low population densities, ecological specialization and low lifetime reproductive effort, the latter being related to their relatively short lifespan (1–6 years) (Jones *et al.*, 2003). All quoll species found in Australia are now listed by the IUCN (Low Risk—Vulnerable), and the two New Guinea species are of unknown conservation status (Jones *et al.*, 2003).

The spotted-tailed quoll (Dasyurus maculatus) is the largest species of quoll, the second largest marsupial carnivore, and is endemic to Australia. Spotted-tailed quolls are found in Tasmania and on mainland Australia, where they persist in fragmented zones within the south-eastern states and far north Queensland. Populations on mainland Australia have experienced a major decline since European settlement, primarily through habitat fragmentation and loss (Jones *et al.*, 2001, 2003), and they are currently listed as Threatened nationally in all states where they occur (Rare: Tasmania; Vulnerable: national, Victoria; Endangered: Queensland).

Detailed information on reproductive biology is necessary for understanding the behavioral life history strategies and ecology of spotted-tailed quolls, and also a priority

for conservation (Temple-Smith, 2003). Some basic information is available on their breeding biology (Fleay, 1935; Fleay, 1940; Settle, 1978; Conway, 1988) and reproductive anatomy (Flynn, 1910,1911; Pearson and De Bavay, 1953), but because there have been no endocrine studies, important information on the frequency, duration and mechanisms of the species' estrous cycle is largely inferred(Croft, 1982; Conway, 1988; Collins *et al.*, 1993).

Most marsupials are solitary (Russell, 1984), but the spotted-tailed quoll is considerably less abundant (Jones and Barmuta, 1998) and less social (Croft, 1982; Collins *et al.*, 1993; Belcher and Darrant, 2004) than other dasyurids. The species also occupies relatively large home ranges and females are territorial (Belcher and Darrant, 2004; Claridge *et al.*, 2005). We hypothesized that female quolls may be induced ovulators to allow increased opportunity for fertilisation upon encountering a male, and that this would be reflected in differences between mated and non-mated estrous cycles.

Most previous research into reproductive endocrinology of marsupials has relied on measurement of plasma progesterone and/or estradiol (reviewed in Tyndale-Biscoe and Renfree, 1987). Species studied include dasyurids such as the kowari and eastern quoll (*Dasyuroides byrnei* Fletcher,1985; *Dasyurus viverrinus* Hinds, 1989), brown antechinus (*A. stuartii* Hinds, 1990) and brush-tailed phascogale (*Phascogale tapoatafa* Millis *et al.*, 1999). More recently, fecal steroid monitoring has been applied as an alternative, non-invasive technique to monitor reproductive cycles in marsupials including the chuditch (*Dasyurus geoffroii*) (Stead-Richardson *et al.*, 2001) and the squirrel glider (*Petaurus norfolcensis*) (Woodd *et al.*, 2006).

The central aim of this study was to characterize the estrous cycle in the spotted-tailed quoll through measurement of plasma progesterone. A second aim was to apply fecal sex steroid analysis as an alternative non-invasive technique for monitoring ovarian cycles in this species as a basis for future applications of such techniques to in situ conservation and captive breeding. This study will contribute to our overall understanding of the diversity of reproductive patterns among the dasyurids, and complements research into the reproductive endocrinology of the closely-related

Tasmanian devil (*Sarcophilus harrisii*), described in a companion paper (Hesterman *et al.*, 2008) (see Chapter 3).

3.3 Materials and Methods

3.3.1 Study animals and husbandry

Ten female spotted-tailed quolls (1–3 years old) were housed at Featherdale Wildlife Park (FWP Doonside, NSW; n = 3) and Trowunna Wildlife Park (TWP Mole Creek, TAS; n = 7) during 2000–2001. Because there were few spotted-tailed quolls in captivity in Tasmania at the onset of the study in May 2000, in July of that year two wild quolls with pouch young were trapped from the Mole Creek district and relocated to TWP under permit. They were captured in wire-cage carnivore traps, aged and inspected for health, sex and status and individually marked by ear tattoo prior to transfer into captivity.

Quolls were maintained under similar conditions at both wildlife parks. They were fed a variety of meats including kangaroo or wallaby, possum, rabbit and chicken, and a variety of beef, mutton, poultry and fish at FWP. Additional items were provided for nutrition and enrichment at TWP. These included a prepared mix of grated carrot, apple, pumpkin seeds, egg and insectivore mix (Wombaroo Food Company, Mt Barker, SA) and, occasionally, commercially available cat biscuits. Water was available ad libitum at both sites.

Study animals were housed either in outdoor enclosures or pens with outside access, so exposed to natural variations in photoperiod, except for two quolls at TWP which were kept indoors under a natural lighting regime for a limited period of approximately three months. Animals kept outside were housed on natural substrate and those indoors were maintained on a wooden floor spread with eucalyptus mulch. All had access to climbing structures, native plants and other natural materials. Dens or nest boxes were available for shelter, and the number of retreats provided met or exceeded the number of animals per enclosure.

3.3.2 Experimental design

Adult quolls were housed individually, due to the species' solitary nature in the wild (Belcher and Darrant, 2004), with the exception of two females with unweaned young. Juveniles were separated from their dams at the beginning of March (prior to the onset of the breeding season) and maintained in mixed sex groups or separately until June. These young were included in the study as spotted-tailed quolls are known to become sexually mature in their first year (Conway, 1988).

Immediately prior to the onset of the breeding season females were placed into a specifically designed mating arena for 2 – 3 d per week. The mating arena consisted of three adjacent wire enclosures (each ~3 m x 5 m x 4 m), with adjoining doors. The female was housed in the central pen and a male was kept in each of the two enclosures on either side of her.

To compare mated and non-mated estrous cycles females at TWP were assigned to different treatments groups during the breeding season (Table 1).

Treatment A: Females permitted full access/housed with males during estrus (n = 4 individuals, 5 estrous cycles).

Treatment B: Females with no physical access to males during estrus (n = 7 individuals, 10 estrous cycles).

To compare estrous cycles within individuals, individuals were assigned to different treatments during successive estrous cycles (STQ #02, #06, #07) or in different years (STQ #01). Four female spotted-tailed quolls from the non-mated group were paired with males during a different estrus.

Table 1

Experimental treatment of captive female Spotted-tailed quolls during the breeding season. Treatment A = paired with male/s during estrus; Treatment B = no physical access to males at estrus. + indicates vaginal smear and asterix (*) full isolation indoors under natural photoperiod. Note quoll #01 was monitored during two consecutive breeding seasons.

	TREATMENT GROUP	
ID #	ESTROUS CYCLE 1	ESTROUS CYCLE 2
01 (Yr I)	A	A
(Yr II)	B^{+}	B
02	B	A^{+}
03	B	-
04	B	B^{+*}
05	B	A^{+}
06	B	B^{+}
07	B^{*}	A^{+}

All females were monitored for behavioral and physical signs of estrus (vocalizations, thickened neck and pouch development (Settle, 1978; Conway, 1988; Collins *et al.*, 1993). Vaginal smears were collected from three females paired with males during estrus, and from three other females during one of their non-mated cycles (Table 1). The first group of females were housed adjacent to males for 2–3 days, and permitted access to males when behavioral observations (vocalizations, crouching) indicated they were receptive (Settle, 1978). The adjoining door between the female and the 'preferred' male (based on the female's soliciting behaviors) was opened, and access to the adjacent male prevented with a large, solid screen. Copulation was confirmed by behavioral observation (observer presence or video recording: cameras positioned to cover both outdoor and nestbox activity), or detection of sperm in the vaginal smear. Pairs were separated after copulation and/or when heightened aggression between them was apparent. Female spotted-tailed quolls are reported to accept several males at estrus (Conway, 1988), so females were immediately returned to the central enclosure, and a new male exchanged for the one with which they had been paired. When a female ceased to show interest in males, she was returned to her individual enclosure. To ensure both groups had similar exposure to males, females from the second group were also

placed into the central arena for an equivalent duration, but were not permitted physical access to males.

3.3.3 Sample and data collection and analyses

Plasma collection

At TWP, during the 2001 breeding season, blood samples were collected when animals were handled to obtain data on reproductive status (see below). Quolls were captured by hand or use of a large net. They were restrained unanaesthetised in a sack during sample collection and examination. A peripheral ear vein was pricked with a disposable Stat-Let© lancet and 75–150 µl blood was collected via a heparinised capillary tube. Samples were taken between 0730 and 0930 or 1500–1700 h except when individuals were being captured for husbandry, when blood was collected opportunistically. Samples were kept at 4 °C until centrifuged; the plasma was frozen (-20°C) until radioimmunoassay. Study animals were bled at ~10 day intervals from May to September. To reduce the potential impact of stress on successful rearing of young, handling and blood sampling of females was minimized during the post-mating period.

Fecal collection

Fecal samples were collected between May 2000–December 2001 (TWP); and October 2000–July 2001 (FWP). To ensure the individual identity of samples when pairs were housed together, small colored plastic beads (1 mm diameter) were mixed into a mincemeat ball and fed to the study animals on the day before sample collection. Frequency of fecal collection varied depending on the time of year and breeding status of individual animals. Immediately prior to and during the mating season (May–September), samples were obtained up to three times per week, whereas during the rest of the year samples were collected weekly. Fecal collection, storage and processing for analysis followed the methods outlined in Hesterman *et al.* (2008) (see Chapter 2).

Additional data and sampling

Vaginal (urogenital) smears were collected from all females paired with males, and also from two non-mated individuals. Smears were obtained from the posterior vaginal sinus by introduction of a small cotton swab through a glass speculum (70 mm length · 3 mm

ø). Smears were air-dried, fixed and then stained with acid fuchsin and toluidine blue (Dix and Billings, 1969). Stained smears were examined for percentage of intermediate (IE) and superficial/cornified epithelials (SE), leucocytes and presence of spermatozoa. Pouches were monitored for condition/presence of young, when not constrained by management.

Plasma progesterone analyses

Plasma progesterone was measured by radioimmunoassay (RIA), as detailed in Hesterman *et al.* (2008) (see Chapter 2). Serial dilutions of quoll plasma ran parallel to the progesterone standard curve. Recovery of cold progesterone had a mean recovery within 10% of expected values. All samples were included in a single assay (n = 74). The intra-assay coefficient of variation was 9.5%.

Fecal sample processing and enzyme-immunoassay of steroids

Lyophilized fecal samples (0.1 g) were mixed with distilled water (0.9 ml) and methanol (4.0 ml). After vortexing and centrifugation, 1.0 ml of the methanol extract was transferred into a new vial, mixed with a $NaHCO_3$ solution and re-extracted with diethylether, following previously described methods (Schwarzenberger *et al.* 2000). Assay buffer was added to the extract residue prior to enzyme-immunoassay (EIA). Immuno-reactive progesterone and estrogen metabolites were assayed using previously established group-specific assays (Schwarzenberger *et al.* 1997). Samples were analyzed for 20α-OH-pregnanes (antibody: 5ß-pregnane-3α-20α-diol 3HS:BSA; trivial name pregnanediol), 20-oxo-pregnanes (antibody: 5α-pregnane-3ß-ol-20-one 3HS:BSA), and total estrogens (antibody: oestradiol-17ß-OH 17-HS:BSA). Preliminary testing showed that, for fecal pregnanes the use of 20α-OH-pregnanes (pregnanediol PgD) was most appropriate for the spotted-tailed quoll, with concentrations being excreted in consistently higher levels than 20-oxo-pregnanes. EIAs were validated by demonstrating parallelism between standard curves and serial dilutions of the fecal extracts, and by showing that fecal values followed the same trend as the values obtained with the plasma progesterone assay The intra- and inter-assay coefficients of variation for the assays were < 10% and <15% respectively.

Terminology

Non-conceptive cycles in marsupials have been described variously as non-pregnant, pseudopregnant or failed pregnant. Following Hesterman *et al.* (2008) (see Chapter 2), the terms "non-mated" and "mated" are used to avoid ambiguity, because some females may have produced young, but lost them prior to pouch checking.

3.3.4 Interpretation of hormone data

Stages of the estrous cycle were defined as the follicular phase (FP), luteal phase (LP), anestrous and inter-estrus (period between beginning of FP to onset of next FP). Baseline values were generated by averaging values of plasma progesterone concentrations obtained from three mature study animals during the non-breeding season (n = 21 samples). For plasma progesterone monitoring, onset of estrous was readily determined because concentrations rose characteristically at pro-estrous, as reported for several other dasyurids (Hinds, 1981; Fletcher, 1985). Increases above the group mean baseline + one SD (*i.e.* 0.07 ng/ml) that were maintained for at least 5 days were considered indicative of onset of the follicular phase (FP). The LP was confirmed when plasma progesterone concentrations increased two SD above baseline levels (i.e. 0.09 ng/ml). Where samples were taken P7d apart, the durations of successive stages of the cycle were calculated by counting the days elapsed between the two samples, halving the result and adding it to the duration of the phase either side. The onset and length of the inter-estrus period were similarly defined, taking the days elapsed between the final two consecutive low plasma progesterone values (LP) and adding half the result to the days before subsequent significant increase (FP) in progesterone.

For fecal steroids, group baseline values were calculated by averaging steroid concentrations in six adults during the non-breeding season (n = 33 samples), as described for plasma progesterone. The FP was defined as the period during which fecal estrogens were elevated above the mean baseline + one SD for the group (i.e. 7.6 ng/g), and remained elevated for at least two consecutive samples. As for plasma progesterone, fecal pregnanes often showed a characteristic pro-estrous pulse that approached mean values. The LP was defined through a sustained increase in progestagen concentrations above the group mean for PgD (i.e. >4456.5 ng/g). The end of the LP was identified by a

decline in pregnane concentrations to below the group mean values for a minimum of 2 weeks.

3.3.5 Sample and data collection and analyses

Plasma collection

At TWP, during the 2001 breeding season, blood samples were collected when animals were handled to obtain data on reproductive status (see below). Quolls were captured by hand or use of a large net. They were restrained unanaesthetised in a sack during sample collection and examination. A peripheral ear vein was pricked with a disposable Stat-Let© lancet and 75–150 µl blood was collected via a heparinised capillary tube. Samples were taken between 0730 and 0930 or 1500–1700 except when individuals were being captured for husbandry, when blood was collected opportunistically. Samples were kept at 4 °C until centrifuged; the plasma was frozen (-20°C) until radioimmunoassay. Study animals were bled at ~10 day intervals from May to September. To reduce the potential impact of stress on successful rearing of young, handling and blood sampling of females was minimized during the post-mating period.

Fecal collection

Fecal samples were collected between May 2000–December 2001 (TWP); and October 2000–July 2001 (FWP). To ensure the individual identity of samples when pairs were housed together, small colored plastic beads (1 mm diameter) were mixed into a mincemeat ball and fed to the study animals on the day before sample collection. Frequency of fecal collection varied depending on the time of year and breeding status of individual animals. Immediately prior to and during the mating season (May–September), samples were obtained up to three times per week, whereas during the rest of the year samples were collected weekly. Fecal collection, storage and processing for analysis followed the methods outlined in Hesterman *et al.* (2008) (see Chapter 2).

Additional data and sampling

Vaginal (urogenital) smears were collected from all females paired with males, and also from two non-mated individuals. Smears were obtained from the posterior vaginal sinus by introduction of a small cotton swab through a glass speculum (70 mm length · 3 mm

ø). Smears were air-dried, fixed and then stained with acid fuchsin and toluidine blue (Dix and Billings, 1969). Stained smears were examined for percentage of intermediate (IE) and superficial/cornified epithelials (SE), leucocytes and presence of spermatozoa. Pouches were monitored for condition/presence of young, when not constrained by management.

2.3.4. Plasma progesterone analyses

Plasma progesterone was measured by radioimmunoassay (RIA), as detailed in Hesterman *et al.* (2008) (see Chapter 2). Serial dilutions of quoll plasma ran parallel to the progesterone standard curve. Recovery of cold progesterone had a mean recovery within 10% of expected values. All samples were included in a single assay (n = 74). The intra-assay coefficient of variation was 9.5%.

2.3.5. Fecal sample processing and enzyme-immunoassay of steroid

Lyophilized fecal samples (0.1 g) were mixed with distilled water (0.9 ml) and methanol (4.0 ml). After vortexing and centrifugation, 1.0 ml of the methanol extract was transferred into a new vial, mixed with a $NaHCO_3$ solution and re-extracted with diethylether, following previously described methods (Schwarzenberger *et al.*, 2000). Assay buffer was added to the extract residue prior to enzyme-immunoassay (EIA). Immuno-reactive progesterone and estrogen metabolites were assayed using previously established group-specific assays (Schwarzenberger *et al.*, 1997). Samples were analyzed for 20α-OH-pregnanes (antibody: 5b-pregnane-3a-20α-diol 3HS:BSA; trivial name pregnanediol), 20-oxopregnanes (antibody: 5a-pregnane-3b-ol-20-one 3HS:BSA), and total estrogens (antibody: oestradiol-17ß -OH 17-HS:BSA). Preliminary testing showed that, for fecal pregnanes the use of 20α-OH-pregnanes (pregnanediol PgD) was most appropriate for the spotted-tailed quoll, with concentrations being excreted in consistently higher levels than 20-oxopregnanes. EIAs were validated by demonstrating parallelism between standard curves and serial dilutions of the fecal extracts, and by showing that fecal values followed the same trend as the values obtained with the plasma progesterone assay. The intra- and inter-assay coefficients of variation for the assays were <10% and <15%, respectively.

Terminology

Non-conceptive cycles in marsupials have been described variously as non-pregnant, pseudopregnant or failed pregnant. Following Hesterman *et al.* (2008) (see Chapter 2), the terms ''non-mated'' and ''mated'' are used to avoid ambiguity, because some females may have produced young, but lost them prior to pouch checking.

3.3.6 Interpretation of hormone data

Stages of the estrous cycle were defined as the follicular phase (FP), luteal phase (LP), anestrous and inter-estrus (period between beginning of FP to onset of next FP). Baseline values were generated by averaging values of plasma progesterone concentrations obtained from three mature study animals during the non-breeding season (n = 21 samples). For plasma progesterone monitoring, onset of estrous was readily determined because concentrations rose characteristically at pro-estrous, as reported for several other dasyurids (Hinds, 1981; Fletcher, 1985). Increases above the group mean baseline + one SD (*i.e.* 0.07 ng/ml) that were maintained for at least 5 days were considered indicative of onset of the follicular phase (FP). The LP was confirmed when plasma progesterone concentrations increased two SD above baseline levels (*i.e.* 0.09 ng/ml).

Where samples were taken ≥7d apart, the durations of successive stages of the cycle were calculated by counting the days elapsed between the two samples, halving the result and adding it to the duration of the phase either side. The onset and length of the inter-estrus period were similarly defined, taking the days elapsed between the final two consecutive low plasma progesterone values (LP) and adding half the result to the days before subsequent significant increase (FP) in progesterone.

For fecal steroids, group baseline values were calculated by averaging steroid concentrations in six adults during the non-breeding season(n = 33 samples), as described for plasma progesterone. The FP was defined as the period during which fecal estrogens were elevated above the mean baseline + one SD for the group (*i.e.* 7.6 ng/g), and remained elevated for at least two consecutive samples. As for plasma progesterone, fecal pregnanes often showed a characteristic pro-estrous pulse that approached mean

values. The LP was defined through a sustained increase in progestagen concentrations above the group mean for PgD (*i.e.*>4456.5 ng/g). The end of the LP was identified by a decline in pregnane concentrations to below the group mean values for a minimum of 2 weeks.

3.3.7 Comparison between plasma and feces

To allow comparison between the profiles of plasma progesterone and fecal progestagen concentrations, temporal alignment of samples was necessary. Because the lag period corresponds with the approximate passage of food (Schwarzenberger *et al.*, 1996), fecal samples were displaced from the plasma results by 24 h—based on the appearance of the colored plastic beads fed to spotted-tailed quolls to individually identify scats.

3.3.8 Statistical analyses

All data are presented as means ± SE, except where indicated otherwise. Student's unpaired t tests were used to compare estrous characteristics and the duration of estrous cycles. Analysis of variance (ANOVA) with Tukey's post-hoc comparison was used to detect temporal changes in grouped data. Plasma progesterone profiles were compared with those of its fecal metabolites using linear regression on log-transformed data. Statistical analyses were performed using SPSS (SPSS Inc. 1998, Chicago IL), Version 13 package.

3.4 Results

Estrous cycles were recorded in all seven female quolls sampled during the breeding season. Longitudinal profiles showed that animals typically underwent estrus twice in each season, and reproductive activity lasted approximately four months, from mid-May until late September.

3.4.1 Estrous cycle characteristics

Mean duration of the estrous cycle varied between treatment groups (Table 2). There was a significant difference in the mean duration of the non-mated and mated estrous cycle ($t_{(11)}$ = -3.221, P = 0.008). This was due to the extended length of the FP in non-

mated females ($t_{(14)}$ = -2.516, P = 0.025). Estrous cycles did not follow each other closely. After the LP, a variable period of ovarian quiescence was observed prior to onset of the next estrous cycle (30.3 ± 6.9 d, range = 7 –59; n = 6). This resulted in an inter-estrus interval of around 2.5 months between successive follicular phases. Intervals were highly variable, both between individuals and between cycles of the same individual.

3.4.2 Plasma progesterone

Mean plasma progesterone concentrations for female spotted-tailed quolls sampled during the breeding period (1.2 ± 0.41 ng/ml) were significantly higher than during the non-breeding period (0.1 ± 0.05 ng/ml) ($t_{(59)}$ =-2.285, P = 0.028). Individual profiles (*e.g.* Fig. 1) demonstrated a biphasic pattern: there was a brief rise (0.97 ± 0.3 ng/ml) in concentrations about one week prior to onset of the LP, accompanied by behavioral estrus, a predominantly cornified vaginal smear and copulations for paired females.

Table 2

Characteristics of the estrous cycle in the spotted-tailed quoll as assessed by changes in fecal (estrogens and progestagens) and plasma (progesterone) sex steroid concentrations. Inter-estrus based on entire cycles only. Significant difference between mated and non-mated cycles indicated by asterix (*) ($P < 0.05$).

	FECES Mean length ± S.E. (Days)	Number of cycles (# individuals)	PLASMA Mean length ± S.E. (Days)	Number of cycles (# individuals)
FOLLICULAR PHASE (FP)*				
Non-mated	19.4 ± 4.0 (range 9 – 39)	12 (6)	15.2 ± 1.4 (range 11 – 18)	6 (4)
Mated	8. 3 ± 1.9 (range 5 – 12)	6 (4)	8.5	1 (1)
LUTEAL PHASE (LP)				
Non-mated	23.5 ± 1.2 (range 18 – 27)	9 (6)	20.7 ± 1.4 (range 19 – 24)	4 (4)
Mated	23. 8 ± 1.7 (range 20 – 28)	4 (4)	-	
ESTRUS CYCLE (FP + LP)*				
Non-mated	41.1 ± 2.3 (range 31 - 52)	8 (5)	35.3 ± 2.5 (range 30 – 40)	4 (3)
Mated	29.2 ± 2.8 (range 23 - 39)	4 (4)	-	
INTER-ESTRUS (FP to FP)	71.1 ± 9.1 (range 49 - 125)	7 (5)	39.5 ± 3.3 (range 30 – 44)	4 (4)

Copulations were observed immediately prior to, during and following this pro-estrous peak in plasma progesterone concentrations. Mating usually occurred over only 1–2 days but was extended up to a week when females accepted two different males at estrus. After the pro-estrous pulse, plasma progesterone concentrations dropped and remained low for several days, but then rose again at luteal onset. During the LP, mean plasma progesterone concentrations were several fold higher (2.18 ± 1.1 ng/ml) than FP levels (0.75 ± 0.02 ng/ ml), with peak values in individual females of up to 8.61 ng/ml.

3.4.3 Comparison between plasma progesterone and fecal metabolites

Fecal progestagens and plasma progesterone during the estrous cycle were significantly positively associated ($P < 0.05$) (regression equation: $y = 0.69 x - 3.10$; $R^2 = 0.72$), and tracked each other (Fig. 1). PgD (20-a- OH-pregnanes) were excreted in consistently higher concentrations than 20-oxo-pregnanes throughout the cycle. Fecal PgD and 20-oxo-pregnanes were significantly associated ($P < 0.01$) (regression equation: $y = 1.08x + 1.17$, $R^2 = 0.73$). Mean PgD concentrations were 3860.2 ± 241 ng/g; mean 20-oxo-pregnanes were 336.67 ± 38.6 ng/g; total estrogens were 13.02 ± 2.4 ng/g.

3.4.4 Fecal steroid monitoring

During the breeding period average fecal sex steroid concentrations in female quolls were significantly elevated compared to during the non-breeding period (PgD non-breeding = 133.5 ± 17.58 ng/g, breeding = 5525.2 ± 1244.77 ng/g ($t_{(158)} = -2.203$, $P = 0.029$); estrogens non-breeding = 4.0 ± 0.74 ng/g, breeding = 17.6 ± 2.70 ng/g ($t_{(156)} = -2.569$, $P = 0.011$)). Group profiles for faecal pregnanediol (PgD) and total estrogens demonstrate the general pattern of sex steroids during the estrous cycle in the spotted-tailed quoll (Fig. 2). Estrogens were in highest concentrations during the FP, approximately one week prior to luteal onset and declined steadily thereafter ($F(3, 85) = 1.147$, $P = 0.235$). There was a significant temporal difference in faecal PgD between days -10 to 30 from luteal onset ($F(3,85) = 2.994$, $P = 0.035$). Faecal PgD had an inverse pattern to fecal estrogens until day 20 from luteal onset, with highest concentrations in the latter part of the luteal phase (days 11–20). Faecal PgD concentrations were markedly lower during days 21–30 after luteal onset ($P = 0.059$).

Non-mated estrous cycle

Individual profiles show that up to three weeks prior to onset of the LP, small pulses of fecal estrogens were apparent, but fecal PgD concentrations remained relatively low (Fig. 3). Fecal progestagen concentrations climbed very rapidly within days after luteal onset and remained elevated during the LP, then declined sharply and approached baseline values between days 25 and 30. During the LP, fecal estrogen levels fluctuated at low concentrations.

All females underwent a second estrus, indicated by elevations in estrogen excretion coincident with an estrus smear (>95% cornified cells). Time elapsed between estrus periods varied, occurring between days 35–40 for most individuals (n = 4), but lasting 65 and 91 days for two study animals. In two females (#04 and #06), there was no subsequent elevation in PgD concentrations following the second FP (Fig. 3).

Mated estrous cycle

Mean concentrations of fecal estrogen were significantly higher in females that were paired with a male during estrus (mated: 26.2 ± 6.37 ng/g, non-mated: 12.4 ± 1.69 ng/g; $t(119) = 2.466$, $P = 0.015$); however, there were no differences in the mean concentrations of PgD between the treatment groups (mated. 6392.7 ± 2255 ng/ g, non-mated: 4585.3 ± 1518 ng/g; $t(121) = 0.690$, $P = 0.491$).

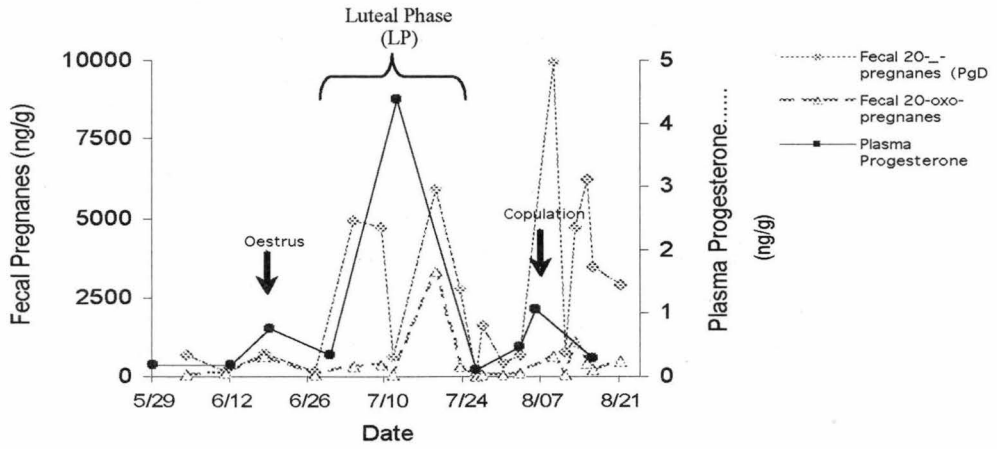

FIG 1

Plasma progesterone (P4) (pg/mL) and fecal progestagen (20-α- and 20-oxo- pregnanes) profiles in a female spotted-tailed quoll sampled during the breeding season. Arrows indicate timing of behavioral oestrus in the first cycle (housed alone) and copulation in the second cycle (paired with male).

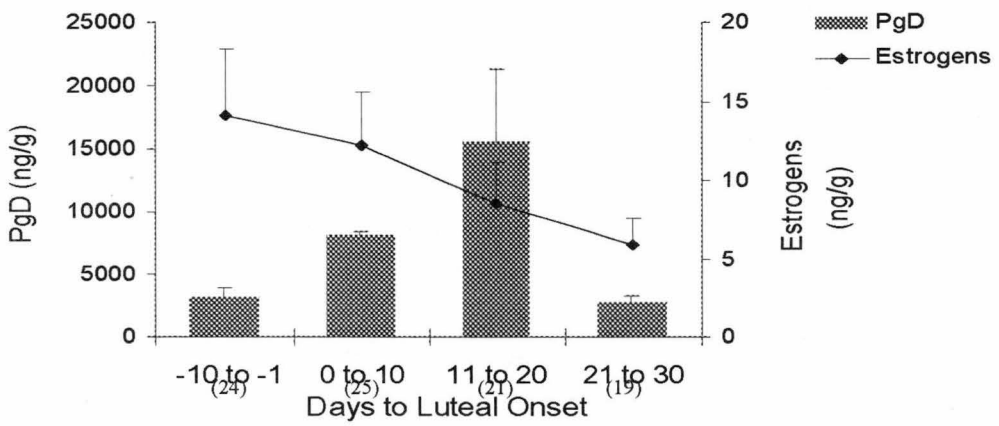

FIG 2

Grouped mean fecal pregnanediol (PgD) and total estrogen concentrations (ng/g) between days -10 to 30 days from luteal onset in seven female spotted-tailed quolls. Sample sizes indicated within bar.

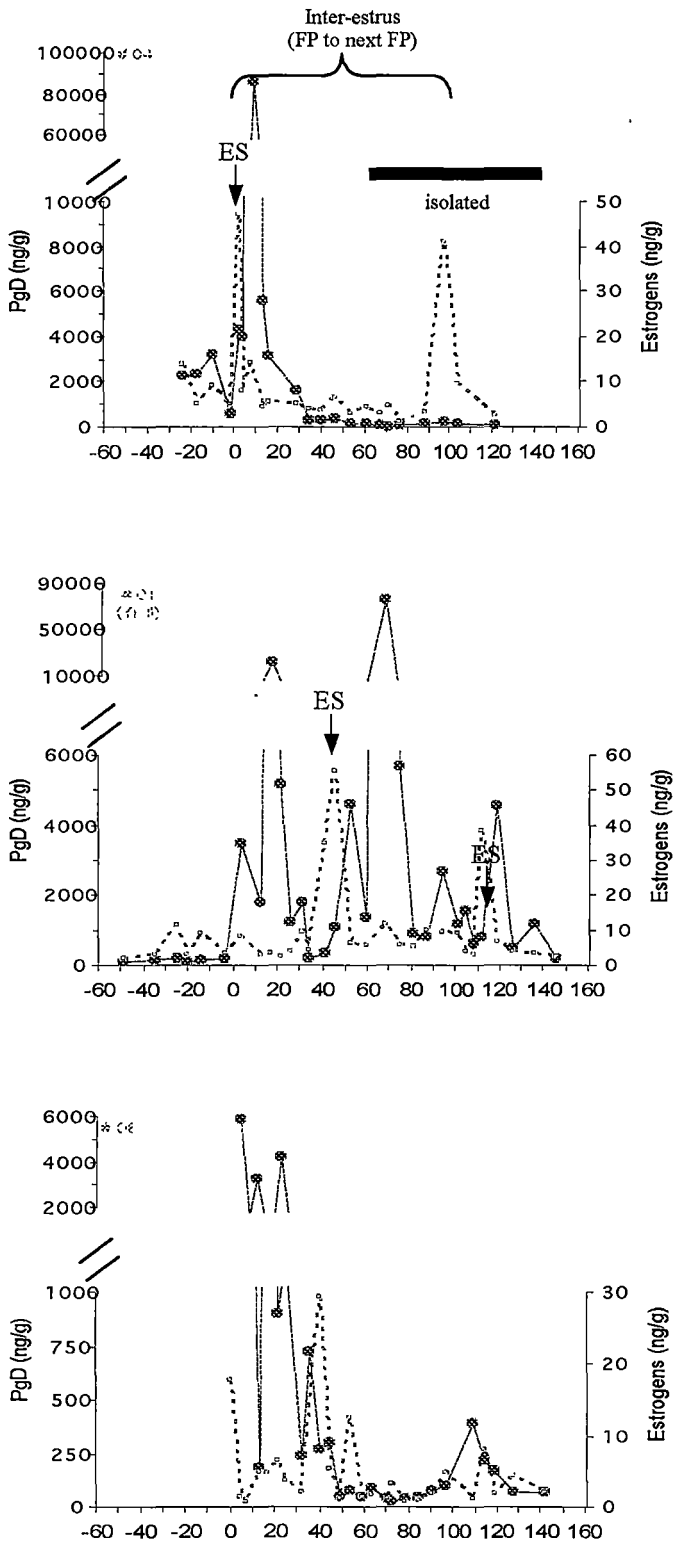

FIG. 3: Longitudinal profiles of fecal pregnanediol (PgD) (•) and total estrogen (□) concentrations (ng/g) during the breeding season in three non-mated female spotted-tailed quolls, aligned from onset of the first luteal phase. Arrows indicate an estrus smear (ES). Solid bar shows period when female #04 (top) was isolated indoors. Female #06 (bottom) was not sampled prior to first luteal onset. Note variable inter-estrus periods between individuals.

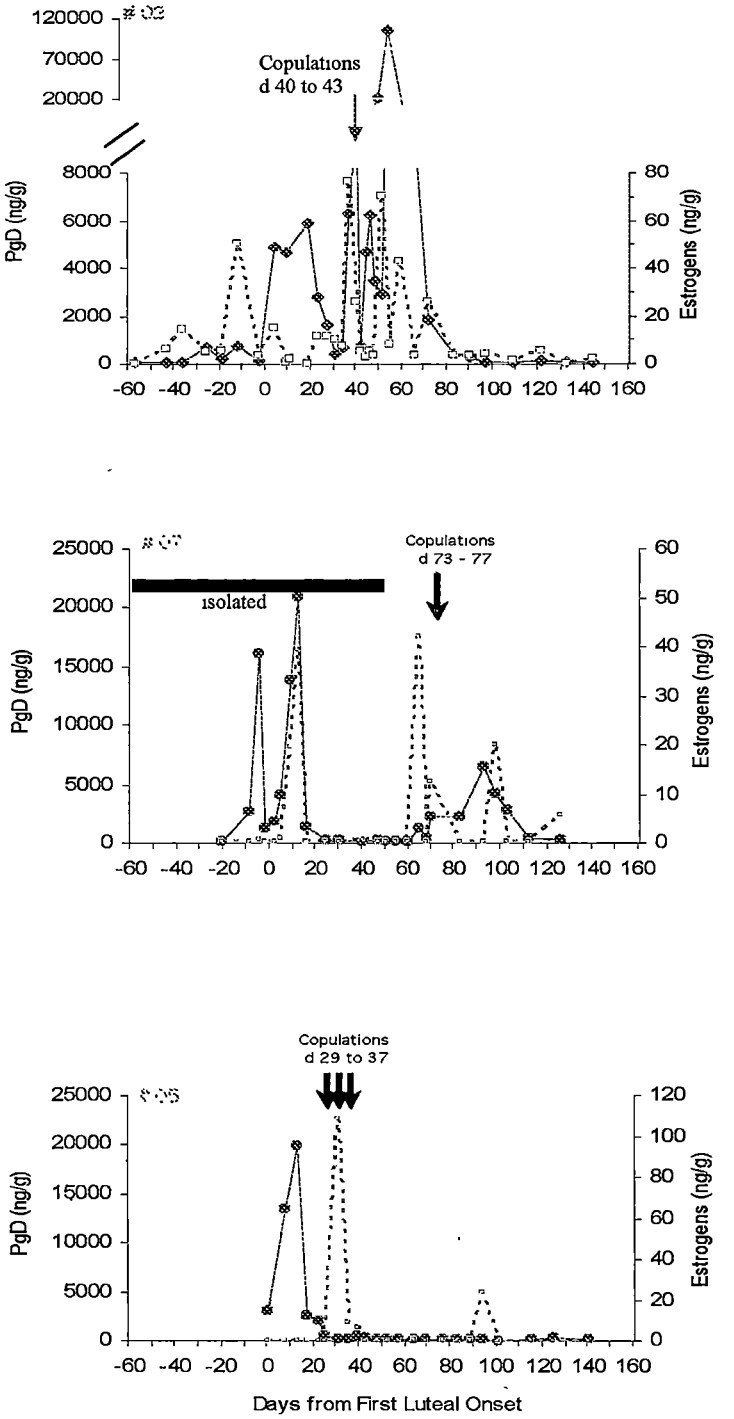

FIG. 4

Longitudinal profiles of fecal pregnanediol (PgD) (•) and total estrogen (□) concentrations (ng/g) in three female spotted-tailed quolls that were mated during their second estrus in the breeding season. Data are aligned from first luteal onset; female (#05) was not sampled prior to that time. Arrows denote periods of copulation. No births were confirmed. Note axis on different scales to account for individual variations in hormone concentrations. Solid bar shows period when female #07 was housed indoors under natural photoperiod.

The four female spotted-tailed quolls mated with males exhibited a similar endocrine pattern to that observed during the non-mated estrous cycle, with a pre-luteal pulse in fecal estrogens during the FP and a sustained increase in PgD during the LP (Figs. 4 and 5). Copulations usually occurred over two–three days, but the interval between mating and luteal onset varied widely between individuals (8.5 ± 3.8 d) in females that ovulated during that cycle. Progestagens peaked around 21 days after first mating at concentrations up to 100-fold higher than those recorded at estrus. Fecal PgD concentrations then dropped precipitously, and were low in three of the four females by day 25 from mating. We could not confirm births for any of the quolls. Individual longitudinal fecal hormone profiles show that major peaks in estrogen excretion were associated with an estrus smear and coincided with times of copulation (Figs. 4 and 5). Anovulatory cycles (no sustained rise in PgD after mating) occurred in three of the four mated individuals. One female exhibited recurrent waves of fecal estrogen excretion 10–20 days apart over an approximate two month period prior to eventual ovulation, despite repeated bouts of mating with two different males (Fig. 5).

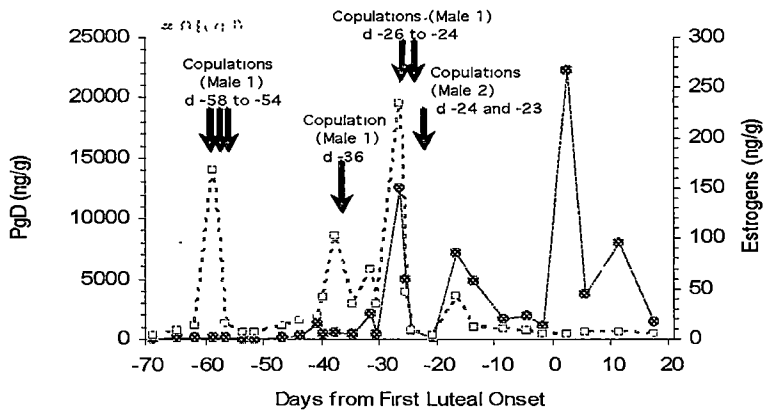

FIG. 5

Longitudinal profile of fecal pregnanediol (PgD) (●) and total estrogen (□) concentrations (ng/g) in a mated female spotted-tailed quoll. Arrows denote episodes of copulation by two different males. Data are aligned from luteal onset; no birth was confirmed.

3.5 Discussion

3.5.1 Characteristics of the estrous cycle

Patterns of plasma and fecal progestagens were significantly associated, indicating that fecal progestagens can be used as a relative measure of concurrent changes in plasma progesterone concentrations. Plasma and fecal steroid monitoring of spotted-tailed quolls showed that although there was some individual variation, on average the estrous cycle lasted approximately 38 days (range 35– 52 d). Settle (1978) reported a 21 day cycle length for the species based only on vaginal cytology. Our endocrine study showed that the average duration of the estrous cycle in spotted-tailed quolls was similar to that of the eastern quoll (Hinds, 1989) and the Tasmanian devil (Hesterman *et al.*, 2008) (see Chapter 2).

Longitudinal profiling confirmed that spotted-tailed quolls are seasonally polyestrous, as is typical for other ıteroparous dasyurids including other quolls (Tyndale-Biscoe and Renfree, 1987; Hinds, 1989) and the devil (Hesterman *et al.*, 2008) (see Chapter 2). Spotted-tailed quoll routinely exhibited two estrous cycles during the reproductive season, although one individual had three estrus periods—an occurrence previously reported by Collins *et al.* (1993). The kowari and the devil also routinely undergo two cycles (Fletcher, 1985; Hesterman *et al.*, 2008) (see Chapter 2). In spotted-tailed quolls the inter-estrus interval (onset of FP to next FP) was ~70 days (range 49 to 125 d), similar to variable at our observations for the devil (Hesterman *et al.*, 2008) (see Chapter 2). Collins *et al.* (1993) reported a shorter, less variable interval of ~50 days (range 36–58 d) for the spotted-tailed quoll based on time elapsed between bouts of female receptivity. In kowari, Fletcher (1985) also found the inter-estrus period to be variable. By comparison, in the eastern quoll estrous cycles are of consistent duration and follow one another closely (Hinds, 1989).

Most marsupials are spontaneous ovulators (Tyndale-Biscoe and Renfree, 1987; Hinds *et al.*, 1996) with very few exceptions: the brush-tailed bettong (*Bettongia penicillata*) (Hinds and Smith, 1992), the American opossum (*Monodelphis domestica*) and the koala (*Phascolarctos cinereus*) (Johnston *et al.*, 2000). In the bettong and opossum, estrus can

be induced by the presence of a male, but the koala is a true 'reflex' ovulator, requiring the physical act of mating to induce a luteal phase. In our study of spotted- tailed quoll, luteal phases occurred in both mated and non-mated cycles, indicating that this species is not an induced ovulator. Interestingly, ovulation occurred in a female housed indoors under complete isolation, but failed to occur in three other unpaired individuals and two mated females, despite episodes of confirmed copulation. However, mated and non-mated cycles did differ significantly in the duration of the follicular phase - being approximately twice as long in non-mated females. This finding indicates that vaginal/cervical stimulation during coitus does play some role in the timing of ovulation in this species.

3.5.2 Pattern of the estrous cycle

The follicular phase

In the spotted-tailed quoll, the follicular phase lasted approximately two weeks, similar to the range reported for other dasyurids (Tyndale-Biscoe and Renfree, 1987; Hinds, 1989; Hesterman *et al.*, 2008) (see Chapter 2). Both plasma progesterone and fecal progestagen concentrations in the spotted-tailed quoll exhibited a relatively low, brief pro-estrous pulse. A similar biphasic pattern has been observed in several other dasyurids including the eastern quoll (Hinds, 1989), kowari (Fletcher, 1985) and Tasmanian devil (Hesterman *et al.*, 2008) (see Chapter 2).

Fecal total estrogens were excreted in highest concentrations during pro-estrous and peaked during the pro-estrous pulse of plasma progesterone/fecal progestagens, as described for the devil (Hesterman *et al.*, 2008) (see Chapter 2). In spotted- tailed quolls and devils, multiple peaks in fecal total estrogens occurred during this period, similar to the wave-like pattern in fecal oestradiol-17ß excretion observed at estrus in the chuditch (Stead-Richardson *et al.*, 2001). During this period of heightened estrogens, we observed cornified vaginal smears and the onset of estrus behaviors, including copulation. Multiple copulations usually occurred over a period of several days, as previously reported for the spotted-tailed quoll (Edgar and Belcher, 1995) and most other dasyurids (Fletcher, 1985; Tyndale-Biscoe and Renfree, 1987; Hinds, 1989; Stead-Richardson *et al.*, 2001; Hesterman *et al.*, 2008) (see Chapter 2).

Following the pro-estrous peak in estrogens and progesterone/progestagens, sex steroid concentrations returned to minimal levels for around a week, prior to a rise associated with onset of the luteal phase. This extended 'ovulatory interval' appears to be characteristic of dasyurids, and has been reported for the eastern quoll (Hinds, 1989), kowari (Fletcher, 1985) and brown antechinus (Selwood, 1980). Mean duration of this 'ovulatory interval' in spotted-tailed quolls (7.1 ± 1.3; range 2 – 12 d) was considerably longer than that reported for those species, but very similar to that of the devil (3–9 d) (Hesterman *et al.*, 2008) (see Chapter 2). This extended timeframe from copulation to ovulation may have a role in permitting matings with several different males, and resultant multiple paternity (Taggart *et al.*, 2003).

The luteal phase

The pattern of progesterone/progestagens during the luteal phase was similar to that observed in other dasyurids (Tyndale-Biscoe and Renfree, 1987; Hinds, 1989; Hinds and Selwood, 1990; Millis *et al.*, 1999; Hesterman *et al.*, 2008) (see Chapter 2). As observed in the Tasmanian devil (Hesterman *et al.*, 2008), fecal estrogen concentrations fluctuated at lower levels during the luteal phase, a pattern that may be related to incomplete suppression of ovarian activity. In most marsupials, plasma progesterone concentrations drop within days after completion of the estrous cycle (Tyndale-Biscoe and Renfree, 1987), and similarly, in spotted-tailed quolls progesterone/progestagen concentrations returned to low levels soon after the end of the luteal phase.

3.5.3 Comparison of the mated and non-mated estrous cycle

As in most other marsupials, there were no differences in the mean duration or amplitude of steroid profiles between mated and non-mated females (Fletcher, 1985; Tyndale-Biscoe and Renfree, 1987; Hinds, 1989, 1990; Hinds and Selwood, 1990; Hesterman *et al.*, 2008) (see Chapter 2). It is unclear why none of the mated female spotted-tailed quoll in this study did not produce young, but profiles did show that copulation was not always followed by ovulation. For uncertain reasons, captive breeding success is low in several dasyurids, including this species (Conway, 1988; Fletcher, 1989; Roberts *et al.*, 1993; Stead-Richardson *et al.*, 2001). Selwood (1983) found that fertilization fails in around 25% of ovulations in the brown antechinus, and

breeding records for spotted-tailed quoll indicate a conception rate of only around 50% in captivity (Collins *et al.*, 1993), although losses of pouch young - especially at early stages of pouch life - are difficult to quantify. Two of the four mated females were only one year old, and a study of wild spotted-tailed quoll suggests that successful breeding may not occur until they reach two years of age (Belcher 2003; Belcher & Durrant 2004).

This study has described the reproductive endocrinology of the female spotted-tailed quoll, *D. maculatus*, documenting the characteristics of the estrous cycle in this Threatened species. It complements our recent research into the closely-related Tasmanian devil (Hesterman *et al.*, 2008) (see Chapter 2) and extends comparisons with other marsupials, as well as demonstrating the usefulness of fecal steroid measurements for monitoring ovarian cycles in carnivorous marsupials. This technique provides a useful, non-invasive method for regular monitoring of reproductive status in spotted-tailed quolls, and may assist in elucidating the reasons for their limited breeding success in captivity.

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CHAPTER 4

POUCH CONDITION AS AN INDICATOR OF BREEDING CONDITION IN THE TASMANIAN DEVIL (*SARCOPHILUS HARISII*) AND SPOTTED-TAILED QUOLL (*DASYURUS MACULATUS*)

4.1 Abstract

Female Tasmanian devils (*Sarcophilus harrisii*) and spotted-tailed quoll (*Dasyurus maculatus*) were monitored to assess changes in plasma progesterone and faecal oestrogens/progestagens, vaginal smears and qualitative changes in pouch appearance during the oestrous cycle. Pouch condition was characterised based on size, colour and secretions, and found to accurately reflect reproductive status, being significantly correlated with changes in both sex steroids and vaginal cytology ($P < 0.05$). During the follicular phase, pouch redness and secretions were maximal, and associated with increased sex steroid concentrations, a karyopyknotic index of >90 % and the onset of copulation. Post-ovulation, pouches became wet and deep and developed a glandular appearance; plasma progesterone/faecal progestagen concentrations remained high and sustained. These changes in appearance were identical during the pregnant and non-pregnant luteal phase. This study demonstrated that pouch appearance is a reliable physical indicator of reproductive status in the Tasmanian devil and spotted-tailed quoll, and provides an alternative non-invasive method for evaluating the ovarian cycle of these threatened species. This technique can be readily applied to monitor individuals in free-ranging or captive populations, and will aid as a practical tool for improved breeding management.

Keywords: Marsupial; Dasyurid; Reproduction; Progesterone; Progestagens; Oestrogens

4.2 Introduction

Measurement of plasma sex steroid concentrations or assessment of vaginal cytology are commonly used to monitor oestrous cycles in marsupials (Tyndale-Biscoe and Renfree 1987). More recently, faecal sex steroid monitoring has been developed for several marsupial species, including the carnivorous dasyurids (Stead-Richardson *et al.* 2001; Paris *et al.* 2002; Bradshaw *et al.* 2004; Woodd *et al.* 2006; Hesterman *et al.* 2008a; 2008b). This contemporary 'hands off' approach confers obvious advantages, but still poses limitations for day-to-day monitoring of reproductive cycles because of the necessity for technical expertise and time required for sample processing. Urinary and vaginal cytology are comparatively intrusive methods, but are accurate and provide immediate results. These techniques are also routinely used to detect oestrus and ovulation (Woolley 1971; Fletcher 1985b; Millis *et al.* 1999; Stead-Richardson *et al.* 2001), but still require a level of operator skill and access to laboratory equipment for analyses.

In some species such as the small dasyurids, changes in body mass provide an alternative indicator of reproductive status (Tyndale-Biscoe and Renfree 1987), and this measure has been correlated with changes in plasma progesterone during the oestrous cycle (Fletcher 1985b). Behavioural cues such as characteristic vocalisations and posturing have also been used to detect oestrus in captive dasyurids (Croft 1982; Fox and Whitford 1982), but this method is very time-consuming (Williams 1990). More simple and practical methods of detecting oestrous are desirable to improve monitoring and management of *in situ* and *ex situ* marsupial populations.

In dasyurids the pouch area typically undergoes marked development during the breeding season, including an increase in size, intense reddening and secretory activity of the tissues (Woolley 1966; 1974; Tyndale-Biscoe and Renfree 1987). For *Antechinus stuartii*, the abundance of urinary epithelial cells at oestrus is accompanied by changes in appearance of the pouch area (Selwood 1982), which suggests this measure could serve as a useful external indicator of reproductive status. O'Donoghue (1911) described these progressive changes in the eastern quoll (*D. viverrinus*), and

demonstrated a relationship between development of the mammary glands and formation/growth of corpora lutea. However, to date, no studies have investigated changes in pouch condition of dasyurids in relation to hormonal variation during the reproductive cycle.

Dasyurid marsupials are vulnerable to extinction because their characteristically short life history which imposes natural constraints on reproductive output (Cockburn 1997; Jones *et al.* 2003). Maintenance of *ex situ* colonies is thereby, an important facet of conservation but intensive management is required to sustain captive founder populations (Jackson 2003a). As with most captive breeding programs, a reliable and robust method of monitoring the females' oestrous cycle is needed to inform decisions such as the timing of mating introductions. This is of particular importance in solitary species such as devils and quolls because inappropriate grouping can lead to serious injury or death, and loss of young (Guiler 1971; Collins *et al.* 1993; Roberts and Hutchins 1993; Jackson 2003a).

Conservation of the world's largest surviving dasyurids - the Tasmanian devil and the spotted-tailed quoll (*D. maculatus*) - is a current priority, with free-ranging populations facing significant threat, primarily from disease (Hawkins *et al.* 2006) or habitat loss (Jones *et al.* 2003), respectively. The devil and spotted-tailed quoll have a lengthy history of failing to breed reliably in captivity, and reviews concede the primary cause is a lack of reproductive information on these species (Williams 1990; Carnio 1993; Jackson 2003a). Detailed information on the reproductive biology and endocrinology of the devil and spotted-tailed quoll has recently become available (Hesterman *et al.* 2008a; 2008b). Like other marsupials (Tyndale-Biscoe and Renfree 1987), both species are seasonally polyoestrous, spontaneous ovulators with equivalent pregnant and non-pregnant oestrous cycles that last approximately one month. Knowledge of the timing and pattern of oestrus and gestation length is helpful, however, further information is required to direct more effective captive breeding. Practical indicators of oestrus and pregnancy have not been established for either the devil or spotted-tailed quoll.

The main aim of this study was to evaluate changes in pouch condition of devils and spotted-tailed quolls in relation to changes in sex steroids and vaginal cytology during the oestrous cycle, and to determine if this is a reliable technique for monitoring reproductive status in these carnivorous marsupials.

4.3 Materials and Methods

4.3.1 Study animals and data collection

Data were collected from 14 female Tasmanian devils (TD) of varying ages (0.5 – 6 yrs old) and seven spotted-tailed quoll (STQ) of varying ages (1 – 4 yrs old) housed at Trowunna Wildlife Park, Tasmania. Details of study animals and husbandry are provided in Hesterman *et al.* (2008a; 2008b). Devils and quolls were restrained unanaesthetised in a sack during physical examination and collection of blood and vaginal smears, and urogenital opening measurements. Animals were cradled in the lap with the hindquarters exposed and positioned toward the handler to facilitate examination. Sampling was conducted during the period encompassing peak breeding activity for that species: data were collected from devils on a twice weekly basis from Jan – Apr, and approximately weekly thereafter to Jun/Jul; and for spotted-tailed quolls at ~10 day intervals from Jun – Sep. Sampling frequency was increased to every 2 – 3 days at the onset of oestrus. To reduce any potential impact of handling stress on breeding success, only limited plasma sampling took place after copulation; sampling resumed if subsequent pouch checks confirmed no young were present. Research was conducted with the approval of University of Tasmania Animal Ethics Committee (permit # A5706).

4.3.2 Pouch assessment and morphometrics

Pouches were monitored for changes in appearance, and checked for the presence of young. Data were collected on qualitative factors *i.e.* size, colour and type/amount of secretion present and the pouch area was scored (0 – 4). Urogenital opening colour was graded on a quantitative scale (white = 1, pink = 2, red = 3). Body weight (to 100g) and urogenital opening measurements (length and width to 0.1mm) were also measured.

4.3.3 Vaginal (urogenital) smears

Smears were obtained from the posterior vaginal sinus by introduction of a small cotton swab through a glass speculum (quolls : 70 mm long x 3 mm ø; devil: 5 mm ø). Smears were air-dried, fixed and stained with acid fuchsin and toluidine blue (Dix and Billings 1969). Stained smears were examined at x 40 magnification. The percentages of intermediate (IE) and superficial/cornified epithelials (SE), leucocytes and parabasal cells were calculated. The karyopyknotic index *i.e.* % cells with pyknotic nuclei, excluding parabasals (Hughes and Dodds 1968), was determined using a total of one hundred cells from five randomly selected fields per slide. In mated females, the presence of spermatozoa was also recorded.

4.3.4 Plasma and faecal collection

A sample of 75-150 µl blood was obtained from a marginal ear vein via a heparinised capillary tube, and centrifuged to recover the plasma, which was stored frozen (-20°C) until radioimmunoassay. Entire faeces were usually collected in the morning (0730 - 0900 hrs) or opportunistically when freshly voided throughout the day, and frozen at -20°C. Plasma and faecal processing and analyses follow methods in Hesterman *et al.* (2008a; 2008b), and are briefly described below.

4.3.5 Plasma steroid analysis

Duplicate aliquots of plasma were assayed for progesterone by radioimmunoassay as detailed in Hesterman *et al.* (2008a; 2008b). Cross-reactivities of the antiserum with other steroids are: 4-pregnen-20β-ol-3-one (1.3%), 4-pregnen-20α-ol-3-one (0.8%), 17α-hydroxyprogesterone (0.6%), deoxycorticosterone (3.3%), corticosterone (0.6%), 11-desoxycortisol (0.4%) and all others (< 0.1%). Assay sensitivity was 3 pg/tube (0.09 ng/ml). Intra- and inter-assay coefficients of variation were 9.5% (n = 9) and 14.8% (n = 9), respectively.

4.3.6 Faecal steroid analysis

Samples were analysed for 20α-OH-pregnanes (antibody: 5ß-pregnane-3α-20α-diol 3HS:BSA; trivial name pregnanediol) and total oestrogens (antibody: oestradiol-17ß-OH 17-HS:BSA), as detailed previously (Hesterman *et al.* 2008a; 2008b). Assay sensitivity

was 2 ng/g both for progestagens and for oestrogens. The intra- and inter-assay coefficients of variation for the assays tested were between 10% and 15%, respectively.

4.3.7 Interpretation of hormone data

Stages of the reproductive cycle determined were anoestrus, oestrus and the luteal phase. These stages have been defined previously, using analyses of group mean concentrations of plasma (progesterone) and faecal (oestrogens and progestagens) sex steroids for the Tasmanian devil (Hesterman *et al.* 2008a) and spotted-tailed quoll (Hesterman *et al.* 2008b).

4.3.8 Statistical analyses

Urogenital opening size was standardised by dividing each measurement by the individual's body weight. All data are presented as the mean ± SE, except where indicated otherwise. Chi-square analysis was used for determining association between pouch or urogenital opening score and reproductive status. One way analysis of variance (ANOVA) with post-hoc Tukey's tests were used to test for significant differences between mean hormone concentrations or karyopyknotic index with pouch condition/score. The significance level was set at $P < 0.05$. Statistics were performed using SPSS (Inc. 1998, Chicago IL, USA).

4.4 Results

4.4.1 Pouch condition

In Tasmanian devils and spotted-tailed quolls, pouch development followed a predictable sequence (Fig. 1a – f; Table 1). Until at least one month prior to onset of the breeding season (TD = Jan; STQ = May), pouches were pale and shallow (Fig. 1a). In subadult devils (< 2yrs of age) and quolls (< 1yr of age) pouch dimensions were considerably smaller (~ 20 – 30 mm diameter, 10 mm depth) (score = 0) than those of adults (~ 50 mm in diameter, > 30 mm deep) (score = 1). Teats were everted and small (< 5 mm) in all females, including individuals that had weaned young from the previous season. Post-weaning there was no difference in pouch appearance between females that had reared one or more litters previously, and those that had never produced young.

At onset of the breeding season study animals showed a marked change in pouch appearance, with the exception of one of the two first year devils. For all other individuals, initially the pouch skin turned pink and the interior began to secrete a pink/red, sticky substance (score = 2; Fig. 1b). During the next stage, the pouch secreted an increasing quantity of the oily exudate and became slightly deeper (score = 3). Production of pigmented pouch 'oil' was more pronounced in devils than in quolls. At times devil pouches contained considerable quantities (> 1 ml) of the red oily exudate, at times developing a crimson ring around the edge (Fig. 1c). The third stage of pouch development was most visibly conspicuous (score = 4). At this time the pouch enlarged noticeably, becoming at least twice as deep (> 50 mm) and with thickened edges. The interior became very vascular and damp; and a thin, clear fluid replaced the red oily exudate (Fig. 1d). After 1 - 2 weeks, the floor tissue became studded with many small, white raised spots (Fig. 1e). These 'studs' developed in the pouches of non-mated and mated females, and were evident at least a week prior to birth in pregnant animals (Fig. 1f), persisting for 2 - 3 weeks in all females (TD: 18.8 ± 3.1d; STQ: 18.4 ± 2.5d).

Following this developmental sequence, the pouch remained enlarged and some pink-red secretions continued to be present. An increase in secretions was later observed and the sequence of changes began again, but this was less marked than at the onset of the breeding season. After the breeding season, for females without young, pouch secretions gradually decreased and dried out then ceased, and the area reduced in size. In females that underwent full pouch development (stage 4), the pouch did not revert to its original size (stage 0) regardless of whether young were produced.

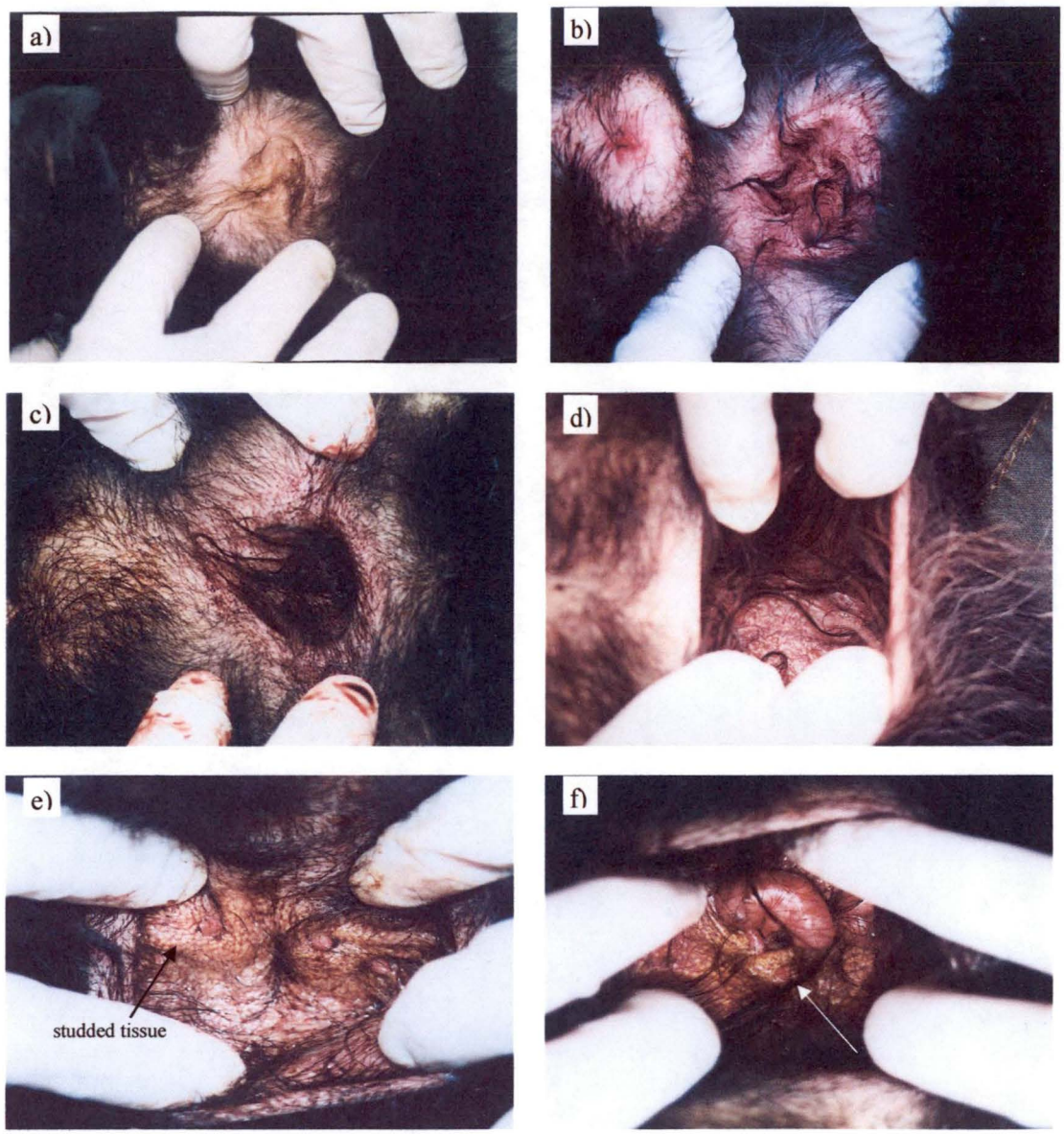

Fig. 1.

Characteristic stages in pouch development of female Tasmanian devils during oestrous. Full descriptions with associated changes in vaginal cytology are provided in Table 1. a) Immature/pre-breeding - pouch is pale and relatively shallow; b) Pro-oestrous - pouch skin is lightly flushed with some red 'greasy' exudate; c) Oestrus - pouch secretes highest levels of red 'grease' (seen pooling on fingertips of glove: note "lipstick" edge; d) Post-ovulation - pouch very enlarged, deep and damp; exudate thin and clear; e) Late luteal phase – tissue wet and glandular with characteristic white "studs"; f) Early lactation - studs persist during early pouch occupation. Pouch young shown ~2 wks of age; note regression of unoccupied posterior teat (indicated by arrow) following loss of another young at early lactation.

Table 1.

Pouch development in female Tasmanian devils and spotted-tailed quolls during the breeding season.

Pouch Score/ Condition	Pouch Description
0 (immature)	Round shape. Pale, small and shallow
1 (adult)	Oval shape. Pale and clean, teats may still be elongated if young only recently weaned; occasional yellow "wax"
2	Skin flushes pink and interior secretes a sticky pink/red, grease-like substance
3	Skin very flushed, marked/copious volumes of thin, red oily secretions produced; area deepens slightly
4	Enlarged: interior very deep and pouch edge thickened; secretion becomes clear and watery leaving interior damp. Pouch hairs thicken and lengthen White studs develop after 1 – 2 weeks, and persist several wks in females without pouch young.
? Post-reproductive (≥ 6 yrs old)	Secretes low - moderate levels of brown, thin/watery liquid

4.4.2 Pouch development and sex steroid concentrations

Longitudinal plasma and faecal endocrine profiles for female devils and quolls demonstrated that changes in pouch condition during the oestrous cycle were associated with significant changes in hormone concentrations (Figs. 2 and 3). In the one year old devil which underwent only minor changes in pouch appearance (minimal secretions, no enlargement), sex steroid concentrations remained basal, confirming this female failed to attain sexual maturity.

Prior to the breeding season, when the pouch was pale and shallow (Fig. 1a; score = 1), concentrations of plasma progesterone and faecal progestagens and oestrogens were low. When the pouch flushed strongly pink to red in colour and began to produce an oily red exudate (Fig. 1b and c; score = 2), there was a concurrent increase in plasma progesterone, faecal progestagens and oestrogens, marking the onset of the oestrous cycle (*i.e.* follicular phase). For females that were paired with males, copulation began when pouch secretions reached maximum (score = 3), and hormone concentrations began to decline (Figs. 2 and 3). The interval from distinctive red exudate production by the pouch to onset of hormonal oestrus was 18.7 ± 2.8 d (range 3 - 32 d) in devils, and 10.9 ± 1.6 d (range 8 – 17 d) in spotted-tailed quolls. Marked deepening of the pouch (Fig. 1d; score = 4) occurred during the luteal phase, when progesterone/progestagen concentrations were highest, and faecal oestrogen concentrations had declined. Individual profiles for devils and quolls showed that substantial enlargement of the pouch began a few days after ovulation. Development of white 'studs' in the pouch appeared approximately one – two weeks after onset of the luteal phase (TD: 13.8 ± 2.3 d, range 8 – 22 d; STQ 9.0 ± 5.4 d, range 4 – 18 d).

In devils, there was a significant association between pouch score and elevated concentrations of plasma progesterone ($F_{4,44}$ = 10.5, $P < 0.01$), faecal pregnanediol (PgD) ($F_{4, 41}$ = 3.45, P = 0.01) and oestrogens ($F_{4, 40}$ = 3.2, P = 0.04) (Table 2). Post-hoc comparisons indicated significantly higher mean concentrations of plasma progesterone/faecal progestagens when the pouch was deep and enlarged (score = 4), during the luteal phase (Fig 1d - f). Similar patterns of hormonal changes and pouch

score (PgD: $F_{3, 16} = 2.16$, $P < 0.05$; oestrogens: $F_{3, 16} = 1.54$, $P = 0.07$) were observed in spotted-tailed quolls.

During inter-oestrous and up to two months after the breeding season, oestrogen and progestagen concentrations were low with minor fluctuations; pouches remained lax and damp and continued to secrete a low quantity of the red exudate. In aged female devils (> 6 years; n = 2), the pouch exudate was brown in colour and thin in consistency (Table 1): endocrine profiles for these individuals indicated missing follicular and/or luteal activity.

4.4.3 Vaginal cytology

For devils, relatively few cells were present in the vaginal smear prior to the breeding season. The cell population consisted mainly of intermediate epithelials (IE) in sexually mature devils (≥ 3 yrs of age), and predominantly parabasals (Pb) in immature females. In spotted-tailed quolls of all ages, the anoestrous smear comprised both IE and Pb cells in females. For both species, there was a proliferation in cell numbers several weeks prior to oestrus: the smear became inundated with IE cells but a low-moderate presence of superficial/cornified epithelial (SE) cells was also observed.

Elevated faecal oestrogen concentrations during the follicular phase (Table 2) were associated with an increased number of mature epithelial cells (IE and SE) in the vaginal smear. Faecal oestrogen concentrations were three-fold higher when the karyopyknotic index was > 90 % (TD: 16.67 ± 5.9 ng/g; STQ: 5.94 ± 1.9 ng/g) than when it was < 50 % (TD: 45.26 ± 10.8 ng/g; STQ: 21.76 ± 10.7 ng/g) (TD: $F_{2, 20} = 1.51$, $P > 0.05$; STQ: $F_{2, 11} = 2.61$, $P > 0.05$). Changes in karyopyknotic index and faecal oestrogen concentrations followed the same pattern during the oestrous cycle, rising steadily during the follicular phase, and then dropping sharply at onset of the luteal phase. The relationship between karyopyknotic index and plasma progesterone/faecal progestagen concentrations was less clearly defined; but in devils, karyopyknotic index and plasma progesterone concentrations were significantly associated ($F_{2, 26} = 4.13$, $P = 0.03$), with highest progesterone concentrations (2.54 ± 0.8 ng/ml) when smears had a low karyopyknotic index (< 50 %).

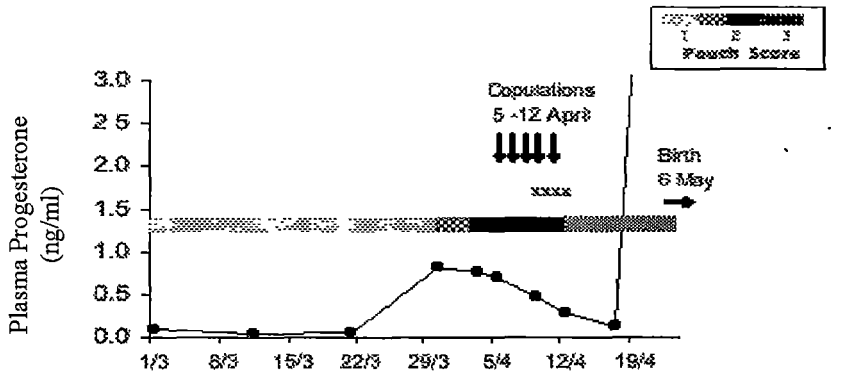

Fig. 2.

Plasma progesterone profile (ng/ml) for an individual female Tasmanian devil, showing associated changes in pouch condition and maturation of vaginal cells during oestrus: 'x' indicates days KI index ≥ 95 %. The pro-oestrous surge in progesterone during the follicular phase is followed by a characteristic nadir prior to ovulation and the major luteal increase (< 10.9 pg/ml, not shown). Birth occurred 24 days after final copulation.

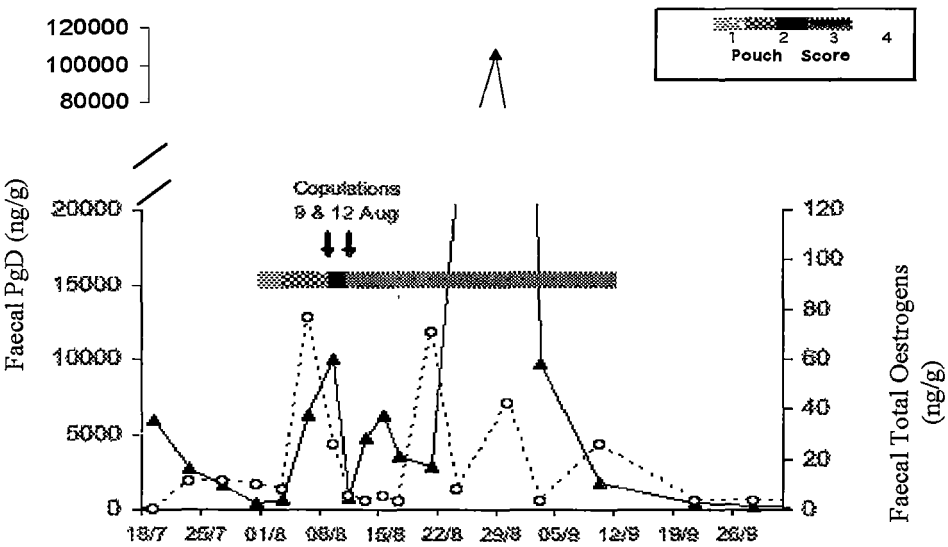

Fig. 3.

Faecal oestrogen (○) and pregnanediol (PgD) (▲) concentrations (ng/g) for an individual female spotted-tailed quoll, showing associated changes in pouch condition at oestrus. No pouch young were confirmed. Terminal portion of previous luteal phase is observed at beginning of profile.

Table 2

Mean concentrations of plasma (progesterone) and faecal (pregnanediol and total oestrogens) sex steroids associated with pouch development in the Tasmanian devil (*Sarcophilus harrisii*) and spotted-tailed quoll (*Dasyurus maculatus*). See Table 1 for description of pouch condition/score. Number of animals sampled in brackets; superscript with different letters indicates significant differences between groups ($P < 0.05$)

Species	Pouch Score	Plasma Progesterone (ng/ml)	Faecal Pregnanediol (ng/g)	Faecal Oestrogens (ng/g)	Stage of Oestrous Cycle
Sarcophilus harrisii	0	0.10 ± 0 0 (12) [a]	152 8 ± 41.5 (12) [a]	7.1 ± 1 6 (12) [a,b]	Immature
	1	0 44 ± 0 1 (9) [a]	499 2 ± 58 2 (10) [a]	17 9 ± 5.1 (10) [a,b]	Anoestrous
	2	0 80 ± 0.2 (11) [a]	750.7 ± 264.6 (10) [a,b]	23 7 ± 5 4 (10) [a,b]	Pro-oestrous; sexually proceptive
	3	1 42 ± 0.6 (11) [a]	1444.9 ± 296.8 (9) [a,b]	32 9 ± 7 5 (9) [a]	Oestrus (follicular phase), sexually receptive
	4	4 72 ± 1 0 (11) [b]	2201 1 ± 456.8 (11) [b]	10 9 ± 2.4 (11) [b]	Post-ovulation; mated and non-mated luteal phase equivalent
Dasyurus maculatus	0	< detection level (1)	-	-	Immature
	1	-	86.4 ± 26 2 (3)	1 1 ± 0 8 (3)	Anoestrous
	2	0 35 ± 0 2 (4)	1390 4 ± 502.2 (6)	16 6 ± 7 7 (6)	Pro-oestrous, sexually proceptive
	3	0 47 ± 0.3 (3)	1307.1 ± 735.4 (5)	10 2 ± 4 7 (5)	Oestrus; sexually receptive
	4	0 74 ± 0 5 (5)	4220 5 ± 1762.6 (6)	4 6 ± 1 4 (6)	Luteal phase; mated and non-mated luteal phase equivalent

4.4.4 Morphometrics

In devils, the urogenital opening enlarged significantly (*length*: $F_{2, 46} = 10.93$, $P < 0.01$; *width*: $F_{2, 46} = 3.79$, $P = 0.03$) during the breeding season and reached maximum size during the luteal phase when progesterone concentrations were high (Fig. 4). For quolls, a similar pattern was observed (anoestrus: 7.5 ± 0.6 x 5.8 ± 1.0 cm/bw; OE: 9.0 ± 0.9 x 6.8 cm/bwt; luteal phase: 8.5 ± 0.9 x 5.7 ± 1.1 cm/bw); however, sample size was insufficient to perform statistical analyses. For devils and quolls, the urogenital opening was pale in non-breeding females, but became pink to red in colour during the oestrous cycle. The change in urogenital opening colour was significantly associated with reproductive condition (assigned using hormone concentrations) in both species (TD: $P < 0.01$; STQ: $P < 0.05$).

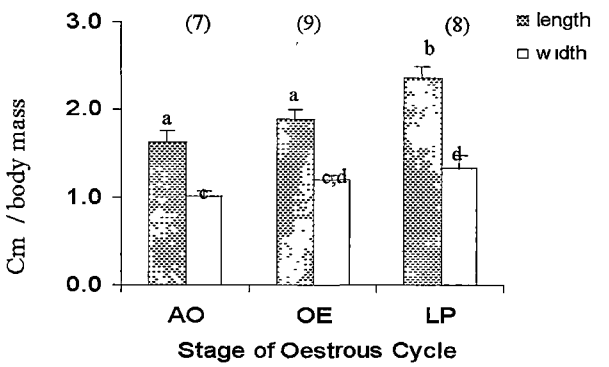

Fig. 4.

Mean urogenital sinus size (standardised cm) during the reproductive cycle in Tasmanian devils. AO = anoestrous, OE = oestrus, LP = luteal phase. Values with different letters indicate significant differences between groups ($P < 0.05$). Numbers of animals for each stage indicated in brackets.

4.5 Discussion

The present study validates pouch condition as a reliable external indicator of reproductive status for Tasmanian devils and spotted-tailed quolls, reflecting the underlying endocrinology of the ovarian cycle in these species. This method provides a simple, accurate and immediate non-invasive tool for assessment of reproductive activity which could benefit monitoring of both captive and free-ranging populations. As females approached their first breeding season the pouch began secreting a pink to reddish oily exudate, signifying the approach of puberty; as documented for other dasyurids (Woolley 1966; 1974). Similarly, in the pubescent brush-tail possum (*Trichosurus vulpecula*), it is the appearance of an orange-brown pouch exudate that heralds first oestrus (Bolliger and Carrodus 1938). As in most other marsupials (Tyndale-Biscoe and Renfree 1987), puberty in devils and quolls was also associated with rapid growth of the pouch, which serves as a useful indicator of sexual maturity.

Our concurrent evaluation of reproductive endocrinology and vaginal cytology demonstrated that readily identifiable changes in pouch appearance are associated with specific stages of the oestrous cycle. Fleay (1935; 1940) noted reddening and development of the pouch of the devil and spotted-tailed quoll during the breeding season, and this feature has been detailed for other dasyurids (Woolley 1966; 1974). O'Donoghue (1911) suggested that similar changes in the eastern quoll pouch result from an increase in size and activity of the cutaneous glands; determining that sweat glands are responsible for producing secretions, whereas hypertrophy of the sebaceous glands results in pouch swelling and enlargement. He suggested that some unknown 'inciting factor' present in blood may be responsible for stimulating glands within the pouch.

In the current study, endocrine monitoring has demonstrated that in devils and spotted-tailed quolls the pouch produces pigmented secretions when oestrogens and progesterone/progestagen concentrations are both elevated. As hormone concentrations continue to rise, there is a concurrent increase in the volume of red pouch secretions. Consistent with this finding, Bolliger and Carrodus (1939b) demonstrated experimentally that oestrogens play a role in the production of pouch pigment in adult

brush-tailed possums. In non-dasyurid marsupials such as the possum, oestrus is associated with elevated oestrogens, and progesterone does not rise until after ovulation (Tyndale-Biscoe and Renfree 1987), implicating oestrogens as the most likely stimulant of this red exudate from the pouch.

Following oestrus, the pouch interior in devils and spotted-tailed quolls became very obviously enlarged and deep - as previously observed in these species (Fleay 1935; 1940). Similar changes have been reported for other dasyurids (Woolley 1974; Fletcher 1985b; Soderquist and Serena 1990; Oakwood 2000). These visible changes were identical in mated and non-mated females, in agreement with Woolley (1974). We confirmed that this stage of pouch development begins soon after ovulation, in association with the major increase in progesterone/progestagens during the luteal phase. Progesterone is a primary agent of pouch enlargement in the brush-tailed possum (Bolliger and Carrodus 1939a), and implicated here as the probable cause of pouch expansion in devils and spotted-tailed quolls. These results also support early speculations that development of the pouch may be linked to development of the corpora lutea (O'Donoghue 1911; Marlow 1961).

The distinctive white spots that appeared later in the cycle, lending a 'granular' appearance to the pouch (Woolley 1966), have also been observed in other dasyurids. O'Donoghue's (1911) histological analysis of the mammary tissues of the eastern quoll determined that these 'studs' are in fact, enlarged sebaceous glands. Similar morphological changes have been observed during pregnancy in several non-dasyurids including the numbat (*Myrmecobius fasciuatus*), wombats (Vombatidae) and the koala (*Phascolarctos cinereus*) (Jackson 2003b; Jackson *et al.* 2003; Power and Monoghan 2003; Finlayson *et al.* 2006). This suggests that pouch appearance may prove to be a useful indicator for monitoring the oestrous cycle in other marsupial species.

The significance of these profound histological developments of the dasyurid pouch during the oestrous cycle is unknown. Given the importance of olfactory signals in dasyurid communication (Croft 1982), it is possible that secretions contain chemosensory properties that serve in mate-attraction or stimulation at oestrus. Male devils have occasionally been observed investigating a female's pouch prior to mating

(HH), and such behaviour has been documented in male macropodids (Tyndale-Biscoe and Renfree 1987). Pouch secretions may also have a role during the peri-parturient stage. O'Donoghue (1911) proposed that later secretions may facilitate cleaning of the pouch by the female prior to giving birth, and there is some evidence that antibacterial properties of pouch secretions may confer some immunity to young marsupials (e.g. Old and Deane 2000).

We also monitored vaginal cytology as an indicator of reproductive condition in the devil and spotted-tailed quoll. In marsupials, proliferation, maturation and secretory activity of the uterine endometrium is correlated with underlying changes in oestrogens and progesterone (Hughes and Dodds 1968; Tyndale-Biscoe and Renfree 1987). The present study showed that the increase in vaginal cell populations and onset of maturation of epithelial cells was mirrored by rapidly rising faecal oestrogen concentrations during the follicular phase: the karyopyknotic index peaked at copulation and declined during the luteal phase in a progesterone-dominant environment. Our findings are consistent with typical changes in vaginal cytology and sex steroid levels in other dasyurids (Selwood 1982; Woolley 1982; Fletcher 1985b; Hinds 1989; Millis *et al.* 1999; Stead-Richardson *et al.* 2001). This confirms that vaginal cytology is a reliable method for determination of oestrus in the devil and spotted-tailed quoll, although the practicality of the technique is limited compared to the simplicity of monitoring changes in pouch appearance.

This is the first study to qualitatively and quantitatively monitor changes in urogenital opening size and appearance during the oestrous cycle in marsupials. We found that enlargement and hyperaemia of the urogenital opening were related to progression of the oestrous cycle and could be used as an external indicator of reproductive condition. Again, there are some limitations to this method because morphological changes are very gradual, and accurate assessment requires careful monitoring of individual animals.

Consistent breeding of devils and spotted-tailed quolls in captivity has continued to prove a challenge (Williams 1990; Carnio 1993; Jackson 2003a), which is of concern given the heightened conservation status and naturally limited reproductive potential of these species (Jones *et al.* 2003). Here we have built on new knowledge of the devil and

spotted-tailed quoll (Hesterman *et al.* 2008a; 2008b) to provide a practical method for monitoring reproduction, but caution that parameters such as age at sexual maturity, timing and length of oestrus and duration of inter-oestrus must also be taken into account to reduce erroneous judgement. Further, detailed studies of pouch development and simultaneous changes in sex steroids are warranted, to determine if correlations could similarly aid in determining reproductive status in other dasyurid and marsupial species.

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CHAPTER 5

LONGITUDINAL MONITORING OF PLASMA AND FAECAL ANDROGENS IN THE MALE TASMANIAN DEVIL (*SARCOPHILUS HARRISII*) AND SPOTTED-TAILED QUOLL (*DASYURUS MACULATUS*)

5.1 Abstract

Improved knowledge of the breeding biology of carnivorous marsupials is warranted given their heightened conservation status. Past studies have focused on smaller dasyurids and little is known of male reproductive physiology in the larger species. This study aimed to characterize the pattern of androgen concentrations in male devils and spotted-tailed quolls and to evaluate fecal steroid measurement as a practical, alternative technique for monitoring reproductive activity. Blood and fecal samples were collected from captive adult devils (n = 6) and adult quolls (n = 8). Plasma and fecal androgen concentrations were significantly positively correlated. In both species there was a significant effect of season on androgen concentrations; and the annual increase preceded female estrus activity. For devils, fecal androgens were elevated during the austral summer: peak concentrations were observed in January - February, and copulation occurred from late February - late May. In quolls, fecal androgen concentrations were highest during austral autumn/winter: the annual increase began in April; copulation occurred from mid-May to early October. The lengthy period of elevated plasma and fecal androgens and protracted period of the year that mating activity continued implies a period of extended spermatogenesis in both species.

Keywords: marsupial; dasyurid; testosterone; hormones; fecal steroids

5.2 Introduction

The Tasmanian devil (*Sarcophilis harrisii*) and the spotted-tailed quoll (*Dasyurus maculatus*) are the largest surviving carnivorous marsupials (max 7 - 12 kg: Strahan 2005) and both species are native to Australia. These closely related dasyurids now co-exist only on the island of Tasmania, and both are on state and national Threatened species lists. Spotted-tailed quolls are naturally rare across most of their range, and are primarily endangered by habitat loss (Jones *et al.* 2003). Devils became extinct on the Australian mainland ~ 400 – 5000 years ago (Jones *et al.* 2003) but remained widespread and common in Tasmania until very recently; the species is currently facing serious risk of extinction from a rapidly spreading, and contagious fatal disease (Hawkins *et al.* 2006; Pearce and Swift 2006). For both species, there is an obvious necessity to maintain self-sustaining captive insurance populations; however, this is particularly challenging in dasyurids because of their naturally short reproductive life span (1 – 3 yrs) and low breeding output (Jones *et al.* 2003). Zoos have had limited breeding success with both species. The current lack of information on their reproductive biology is a major factor in these low success rates (Williams 1990; Carnio 1993; Jackson 2003a).

Despite a strong research focus on the breeding biology of the smaller carnivorous marsupials (Tyndale-Biscoe and Renfree 1987), until recently only basic life history variables were available for the devil and for the spotted-tailed quoll (Fleay 1952; Guiler 1970a; Settle 1978; Manserg 1984; Belcher 2003). Following mating and a short gestation (~ 3 weeks) a single, relatively small litter (4 or 6 young, respectively) are weaned in spring (Fleay 1935; 1940; Green 1967; Guiler 1970a; Belcher 2003). Dasyurids typically have a well regulated annual breeding period (McAllan 2003), yet in the devil and spotted-tailed quoll there is some intriguing evidence that out-of-phase breeding can occur in the wild (Guiler 1970a; Green and Scarborough 1990; Körtner 2006). We have recently characterized the estrous cycle in female devils and spotted-tailed quolls (Hesterman *et al.* 2008a; 2008b), but there is still no published information on the endocrinology of male reproduction for either species.

Dasyurids exhibit a range of reproductive strategies, relating in part to the timing and frequency of female oestrous cycles and also the timing and extent of male effort (Lee *et al.* 1982; Lee and Cockburn 1985). Documenting male androgen rhythms is a necessary step towards understanding breeding synchrony between the sexes. The annual pattern of plasma testosterone concentrations has been reported for a range of male marsupials (reviewed in Tyndale-Biscoe and Renfree 1987) including several other quoll species (Bryant 1986; Schmitt *et al.* 1989), phascogales (*Phascogale*) (Bradley 1987; Millis *et al.* 1999), and the well-studied dunnart (*Sminthopsis*) and marsupial mouse (*Antechinus*) (McDonald *et al.* 1981; Wilson and Bourne 1984). Such studies have traditionally measured concentrations of total androgens or testosterone in plasma. An obvious advantage of developing a non-invasive method for measurement of androgens is to circumvent potential problems caused by the stress of repeated handling, which has been reported to depress peripheral testosterone concentrations in several marsupial species (Lincoln 1978; Curlewis and Stone 1984). Despite the popularity of non-invasive sex steroid measurement in eutherian mammals (reviewed in Lasley and Kirkpatrick 1991; Schwarzenberger *et al.* 1996) and application of these techniques to monitoring reproduction in female marsupials (Stead-Richardson *et al.* 2001; Paris *et al.* 2002; Oates *et al.* 2004; Hesterman *et al.* 2008a; 2008b), there is only one published study on male marsupials (Hamilton *et al.* 2000), an investigation of fecal androgens in southern hairy-nosed wombats (*Lasiorhinus latifrons*).

The primary aims of the present study were: 1) to characterize the annual pattern of androgen concentrations in male devils and spotted-tailed quolls; and 2) to evaluate fecal steroid monitoring as an alternative, non-invasive technique for monitoring testicular rhythms in these dasyurid species.

5.3 Materials and Methods

5.3.1 Study Animals and Husbandry

Samples were collected from six adult male devils (2 – 8 yrs old) and eight adult male spotted-tailed quolls (1 – 4 yrs old) housed at Trowunna Wildlife Park (TWP, Mole Creek, Tasmania) between May 2000 and December 2001. All devils and one quoll were from an already established captive population. Seven male quolls were trapped

from the wild between August 2000 – April 2001, and relocated to the park under permit. They were captured in wire-cage carnivore traps, aged, sexed, inspected for health, and individually marked by ear tattoo prior to being brought into captivity.

Devils and quolls were fed a natural meat-based diet consisting mainly of kangaroo or wallaby and occasionally possum or rabbit. Spotted-tailed quolls received additional items including a prepared mix of grated carrot, apple, pumpkin seeds, egg and insectivore mix (Wombaroo Food Company, Mt Barker, SA). Additional enrichment food items provided less often included commercially available brands of dog or cat biscuits. Water was available *ad libitum*.

Study animals were housed either in outdoor enclosures or pens with outside access, except for two quolls, # 02 and # 07 - which were kept indoors under a natural lighting regime for six - nine weeks respectively, for purpose of display. Animals kept outside were housed on natural substrate; those indoors were maintained on a wooden floor spread with eucalyptus mulch. All had access to climbing structures, native plants and other natural materials. Dens or nest boxes were available for shelter, and the number of retreats provided was greater than or equal to the number of animals per enclosure.

Grouping of the study animals varied during the year according to accepted husbandry management for the species. Male devils were housed individually during the breeding season (Jan – Jun) but were maintained in mixed sex groups at other times of the year. Spotted-tailed quolls were routinely housed individually because of this species' more solitary nature (Belcher and Darrant 2004). Three of the male devils (3/6) and four of the male quolls (4/8) were paired with individual females for breeding purposes (Table 1) as required by husbandry at the captive facility. Pairing duration was dependent on female receptivity. Following mating and/or lack of interest or increased aggression toward males, females were removed to their individual enclosures. Estrus periods of female devils and quolls were determined through endocrine monitoring, as part of concurrent studies; copulations were confirmed by behavioural observation (observer presence/video monitoring within dens), or detection of sperm in a vaginal smear. (Hesterman *et al.* 2008a; 2008b).

5.3.2 Plasma collection

Samples were taken between 1500 – 1700h to reduce potential complications of diurnal variation in plasma testosterone concentrations. Animals were captured by hand or with the use of a large net, and restrained unanaesthetised in a sack during sample collection. Blood was collected within five min of capture. A peripheral ear vein was pricked with a disposable Stat-Let© lancet and 50 -100 µL blood was collected via a heparinised capillary tube. Samples were kept at 4°C until centrifuged later that day, and the plasma separated and stored frozen (-20°C) until radioimmunoassay. Plasma collection focussed on the period encompassing the main breeding season for each species. Blood was collected from five of the devils (2 – 4 yrs old) at intervals of ~ 7 - 10 days during late Jan – July 2001, but not from the single, aged individual (8 yrs old) (#126) (Table 1). Quolls were bled at ~3 – 4 week intervals during Apr – Sep 2001; due to the nervous disposition of this species (HH, pers. obs) , blood sampling was limited to four males only.

Table 1

Details of pairing and breeding activity in male Tasmanian devils (*S. harrisii*) and spotted-tailed quolls (*D. maculatus*).

Species	Studbook / ID #	Paired For Breeding	Pairing Period*
T Devil	126	No	
	203	Yes	22 - 25 May
	272	Yes	21 Feb - 22 May
	295	No	
	329	No	
	666	Yes	23 Mar
S T Quoll	1	Yes	18 - 21 Jun; 16 - 18 Jul
	2	Yes	18/19 Jul, 9/10 Aug year 2
	3	Yes	12 Aug
	4	No	
	5	Yes	18/19 Jun; 1 - 3 Oct
	6	No	
	7	No	
	8	No	

* Range of dates during which males were housed with estrus females

5.2.3 Fecal collection

Fecal samples were collected weekly from all study animals. To identify individual samples when animals were housed together, small coloured plastic beads were mixed into a mincemeat ball and fed to study animals the previous day. Entire fecal samples were collected during morning servicing (0730 - 0900 hrs) or opportunistically when freshly voided throughout the day. When several scats were available from the same individual, the most visibly fresh sample was selected. Samples were placed in zip-lock plastic bags and stored at -20°C for later processing. Frozen samples were freeze-dried (Dynavac FD16, Dynavac High Vacuum Pty Ltd., Victoria, Australia) and sieved through 1mm^2 plastic mesh to remove fur, bones and other fibrous or undigested matter; the screened feces were refrozen until analysis.

5.3.3 Sample analyses

Plasma

Plasma androgen was analysed using an already-established radioimmunoassay procedure (Pankhurst and Conroy 1987). In brief, duplicates of 50 µL of plasma were extracted in 1mL ethyl acetate (AnalaR grade Merck Pty, VIC, Australia) by vortexing and the sealed tubes left to incubate at room temperature for 90 min to maximise extraction. Samples were centrifuged for 5 min at 3000 rpm, and the solvent was recovered and evaporated under air. Evaporated plasma extracts were reconstituted in 200 µLphosphate buffer (0.05 M containing 0.1% gelatine) and vortexed prior to radioimmunoassay (RIA). A serial dilution (range 3 – 400 pg/mL in buffer) of testosterone standards was prepared (T-1500 Sigma-Aldrich Pty, Ltd, Missouri, U.S.A.) and replicate 200 µL of each dilution used to create a standard curve. 200 µL of [^{3}H] testosterone ([1,2,6,7] (TRK402 Amersham Biosciences, NSW, Australia) in assay buffer containing ~3000 cpm was added to tubes containing standards or reconstituted sample extracts. 200 µL of a polyclonal testosterone antiserum (Bioclin, Cardiff UK) diluted 1:50 000 in phosphate buffer was added and tubes were incubated at room temperature overnight. The antibody was donated by N. Pankhurst and cross-reactivities were: testosterone 100 %, progesterone 0.086 %, pregnenolone 0.004%, 17α-hydroxypregnenolone <0.003%, 17α-hydroxyprogesterone 0.003%. The cross-reactivity

of dihydrotestosterone was not assessed; therefore results are reported as plasma androgens. The assay was cooled on ice for 10 min prior to addition of 200 µL dextran-coated charcoal (0.125%) suspension. Tubes were incubated for 10 min on ice, before centrifuging for 10 min (3000 rpm) at 4°C. The supernatant was recovered from each tube by decanting, and counted in 4 mL scintillation fluid (Ecolite, MP Biomedicals, Inc. California, USA) for 3 min in a Beckman Coulter Counter LS 6500.

To determine extraction efficiencies, 5000 cpm [^{3}H] testosterone (Amersham Biosciences, Australia) was added to individual plasma samples before extraction. Mean recovery of radioactive testosterone was 94.0 % (± 3.3) for devils and 98.5 % (± 1.5) for spotted-tailed quolls. Serial dilutions of devil and quoll plasma ran parallel to the testosterone standard curve. Assay sensitivity, determined as the least measured dose of testosterone, was 0.06 ng/mL plasma. Recoveries of added steroid were determined by spiking pooled devil plasma with authentic testosterone (12, 25, 50, 100, 200 ng/mL) which yielded a mean recovery within 10% of expected values Intra- and inter-assay coefficients of variation were 8.4% (n = 10) and 14.6% (n = 3), respectively. All samples from one individual were included in a single assay.

Feces

Feces were extracted as previously described (Hesterman *et al.* 2008a). In brief, 0.1g lyophilized sample was mixed with 10 mL of 90 % ethanol (AnalaR, BDH, Poole, England), and ~5000 cpm tritiated testosterone (Amersham Biosciences, Australia) was added to each tube to determine individual recoveries. The suspension was boiled for 20 min and then samples were centrifuged for 20 min at 2500 rpm. The liquid fraction was recovered and the remaining pellet resuspended in 5 mL 90% ethanol, vortexed, then re-centrifuged for 15 min; this supernatant was also recovered. The supernatants were combined, evaporated and cooled prior to adding 1 mL of phosphate buffer (PBSG 0.05 M pH 7.4; 0.1% gelatin) to dissolve the extract.

For the assay, 50µL [^{3}H] testosterone (~5000 cpm) (Amersham) in ethanol was added to tubes containing testosterone standards (range 3.12 – 200 pg/50 µL in ethanol; Sigma-Aldrich Pty, Ltd, Missouri, U.S.A) or tubes to receive samples, and evaporated. 200µL of extracts diluted (1:20) in phosphate buffer (PBSG 0.05 M pH 7.4; 0.1% gelatin) were

added to the sample tubes. The Sirosera (North Ryde, NSW, Australia) testosterone antiserum (C-6050) was raised in sheep, and cross-reacts with testosterone (100%), androsten-3β, 17β-diol (30%), and 5α-dihydrotestosterone (31%), all others (≤ 0.1%). 100μL of antiserum diluted 1:46 000 in assay buffer was added to reconstituted extracts and to standards, which received a further 200 μL buffer to bring them up to an equivalent volume. The tubes were vortexed briefly, then incubated at 4°C overnight. Unbound steroid was separated by addition of 500 μL dextran-coated charcoal (0.125 %). Tubes were incubated for 15 min on ice, before centrifuging for 15 min (1500g at 4°C). 300 μL of each supernatant was counted in 2.5 mL scintillation fluid (Ecolite, MP Biomedicals, Inc. California, USA) for 5 min in a Beckman Coulter Counter LS 5801.

Mean recovery of radioactive testosterone was 87.7 % (± 0.6 SE) for devils and 86.2 % (± 0.4 SE) for quolls. Results indicated that the dietary content of bone, frequently consumed by both species contributed to variability between samples. Serial dilutions of devil and quoll plasma and feces ran parallel to the testosterone standard curve. Assay sensitivity, determined as the lowest detectable dose of testosterone, was 2 ng/g feces. Recovery of exogenous testosterone (12.5 – 200 pg/tube) was within 10% of expected values for both species (7.4 ± 4 S.E. TD; and 6.5 ± 4 S.E. STQ). Intra- and inter-assay coefficients of variation, determined by including pooled fecal samples, were 11.5% and 14.4%, respectively.

5.3.4 Comparison between plasma and fecal androgens

To allow comparison between the patterns of plasma and fecal androgens, samples were temporally aligned to account for the lag time of fecal steroids (Schwarzenberger *et al.* 1996), which is considered to approximate the passage of digesta (Lasley and Kirkpatrick 1991; Schwarzenberger *et al.* 1996). Based on the time to appearance of physical indicators (small, colored plastic beads) fed to animals to individually identify scats, fecal results were therefore displaced from the plasma results by 24 h.

5.2.6 Data handling and statistical analyses

Data are presented as means ± SE, and were analysed a) by month and b) by season. The austral year was divided into four seasons: December - February (summer), March

- May (autumn), June - August (winter) and September - November (spring). Analyses of variance (ANOVA) were used to detect temporal changes in hormone profiles, and differences in hormone concentrations between seasons, accordingly. This was followed by Tukey's post-hoc comparisons to identify significant differences between groups. Student's unpaired t-test was used to compare hormone concentrations between mated and non-mated males. For regression analysis was data were log-transformed to compare the profiles for plasma androgens and fecal androgen metabolites. The level of significance was set at $P < 0.05$. Statistical analyses were performed using SPSS (SPSS Inc. 1998, Chicago IL), Version 13 package.

5.4 Results

5.4.1 Comparison between plasma and fecal androgens

Representative individual profiles (Figures 1a,b) demonstrate the relationship between plasma and fecal androgen concentrations in devils and quolls,. Patterns of plasma androgen and its' fecal metabolites closely tracked each other: peaks and troughs were well matched. There was a significant positive correlation between plasma and faecal androgens for both species (devils: $P = 0.03$; $y = 0.47x + 0.98$, $R^2 = 0.376$) (quolls: $P = 0.02$; $y = 0.89x + 1.90$; $R^2 = 0.384$). This provided confidence that the pattern of faecal androgens represented concurrent changes in plasma androgen concentrations.

5.4.2 Tasmanian devils

Across all samples, mean concentrations of plasma and fecal androgens were 0.55 ± 0.1 ng/mL (range 0.06 – 1.86 ng/mL), and 14.08 ± 0.6 ng/g (range 2.50 – 49.35 ng/g), respectively. Fecal androgen concentrations were significantly higher in the austral summer compared to all other seasons ($F_{(3, 251)} = 11.506$, $P < 0.001$) (Table 2). Monthly profiling of grouped data revealed the temporal pattern of excretion ($F_{(11, 243)} = 6.340$, $P < 0.001$) (Fig. 2a). Peak fecal androgen concentrations were recorded in January ($P < 0.05$ for Apr-May, Jul- Oct, Dec) and February ($P < 0.05$ for Apr – Dec) then declined slowly but steadily over the next three months, and remained below the mean between July and October Monthly plasma means showed a similar trend to changes in fecal androgens

over the breeding period, with highest concentrations in February (0.65 ± 0.1 ng/mL) declining steadily toward April (0.35 ± 0.1 ng/mL).

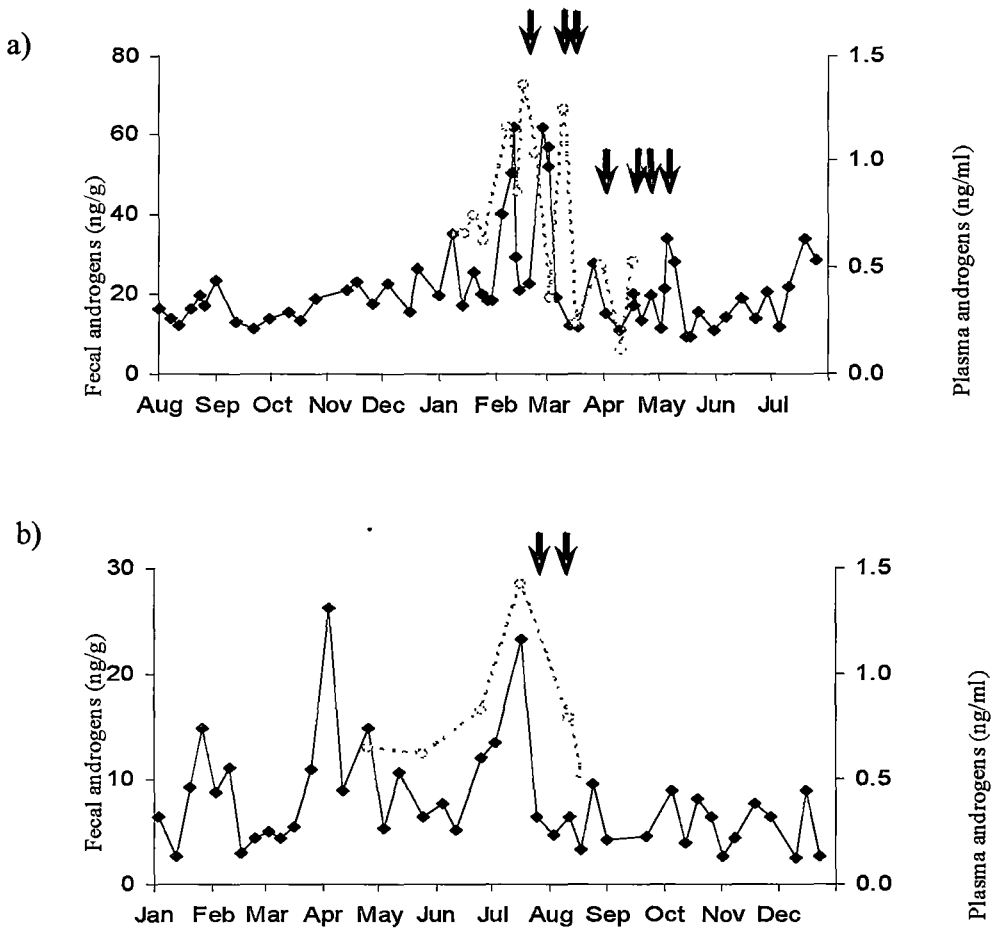

Fig. 1.

Longitudinal profiles (mean ± SE) of fecal (◆) and plasma (○) androgen concentrations in an individual male a) Tasmanian devil; and b) spotted-tailed quoll. Each arrow represents onset of copulations with a different female. Note differences in months between graphs.

Breeding activity took place between February and late May (Table 1; Fig. 2a). For the three male devils given access to females, the period of pairing and copulation was brief (< 3 days) in accordance with the limited period of female receptivity (Table 1). Seven of these ten females produced young; births occurred between 23 March - 22 June 2001. In the paired males, peaks in plasma and fecal androgen concentrations were associated with exposure to estrous females and copulation, but also occurred independent of mating activity (*e.g.* Fig.1a.). Similar fluctuating concentrations were observed in

solitary males throughout the breeding season. There was no significant difference in the pattern of fecal androgens over time ($F_{(11, 231)}$ = 1.036 P = 0.047) or the concentrations of fecal androgens between these two groups ($t_{(253)}$ = 1.166, P = 0.245).

Table 2

Seasonal changes in mean (± SE) fecal androgen concentrations (ng/g) in male Tasmanian devils (n = 6) and spotted-tailed quolls (n = 8). Values with different letters indicate significant differences (P < 0.05) between seasons for each species.

Season	Devils	Number of samples	Quolls	Number of samples
Summer	19.3 ± 1.43 [a]	60	7.8 ± 0.63 [c]	41
Autumn	13.7 ± 0 94 [b]	83	10.7 ± 0.94 [d]	40
Winter	11.7 ± 0.74 [b]	70	9.1 ± 0.50 [cde]	52
Spring	11.4 ± 0.80 [b]	42	7.9 ± 0.49 [ce]	75

5.4.3 Spotted-tailed quolls

Mean concentrations of plasma and fecal androgens across all samples were 0.76 ± 0.1 ng/mL (range 0.40 – 1.42 ng/mL), and 8.90 ± 0.3 ng/g (range 2.52 – 44.33 ng/g), respectively. Fecal androgen concentrations were significantly higher in austral autumn (P < 0.05) than in summer or spring ($F_{(3, 254)}$ = 3.770, P = < 0.01) (Table 2). There was a significant trend in the monthly pattern of fecal androgens ($F_{(11, 246)}$ = 2.067, P = 0.023). Concentrations were low from January – March, increased in April, and peaked by May (Fig. 2b.). Fecal androgen concentrations declined somewhat in July, but remained elevated above the overall mean values until at least August. Plasma means showed a similar trend to faecal androgen concentrations, being increased at sampling onset in April/May (0.67 pg/mL ± 0.10 for each month) compared to concentrations measured in August/September (< 0.5 ng/mL).

Breeding activity in quolls was observed from mid-May until early October (Table 1; Fig. 2b). Episodes of pairing and copulations usually lasted for only around 2 days (Table 1). None of these matings resulted in confirmed births. Individual male profiles showed that major peaks in plasma and fecal androgen concentrations were not strictly associated with pairing or mating activity (*e.g.* Fig.1b.). There was no difference in the concentrations of fecal androgens in paired and non-paired males ($t_{(243)}$ = -1.71, P = 0.08) or the monthly pattern of fecal androgen concentrations ($F_{(11, 54)}$ = 0.669 P = 0.761).

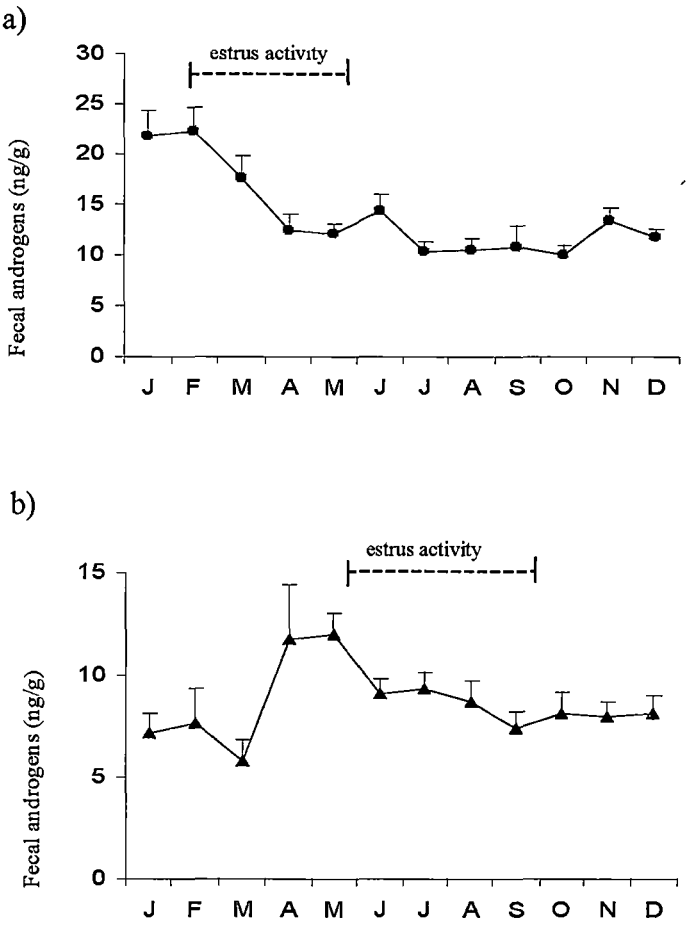

Fig. 2.

Mean (± SE) monthly fecal androgen concentrations in a) male Tasmanian devils (n = 6) and b) male spotted-tailed quolls (n = 8). Dashed line indicates period of estrus activity in females, confirmed by ovarian steroid monitoring (Hesterman *et al.* 2008a; 2008b).

5.5 Discussion

This research has provided the first longitudinal study of fecal androgens in male marsupials and characterised the annual pattern of androgen concentrations in the male Tasmanian devil and the spotted-tailed quoll. Faecal steroid monitoring was proven to represent a useful alternative, non-invasive technique that accurately reflected longitudinal patterns and seasonal concentrations of circulating androgens. The study confirmed that devils and spotted-tailed quolls undergo an annual change in androgen concentrations associated with reproduction that is similar to that seen in other temperate, seasonally breeding marsupials.

5.5.1 Plasma and fecal androgen monitoring

Plasma androgen concentrations were similar between devils and spotted-tailed quolls but although basal levels were comparable to those in other species, peak concentrations were considerably lower than reported for a range of male mammals including other dasyurids (reviewed in Tyndale-Biscoe and Renfree 1987; McAllan 2003). Concentrations were, however, within with the range measured in wild devil (Pemberton 1990, HH unpublished data) and spotted-tailed quoll populations [HH unpublished data]. Pemberton (1990) found that devils of both sexes have consistently elevated plasma corticosteroid levels, and speculated that suppression of androgens may occur in this species as a result of constant social stress. In our study, captive males were either housed alone or kept in small, apparently compatible groups. We suggest that the relatively low mean plasma testosterone concentrations in the closely related devil and spotted-tailed quoll represent an intrinsic difference from other marsupials studied to date.

Mean fecal androgen concentrations were higher in devils (14.06 ± 0.6 ng/g) than in quolls (8.90 ± 0.3 ng/g), but the overall range was very similar between the two species. Although fecal steroid monitoring has been applied to the measurement of androgens in a variety of eutherian species (reviewed in Schwarzenberger *et al.* 1996), the only comparable work on male marsupials is that of Hamilton *et al.* (2000), who used plasma and fecal androgen assays to assess seasonality in wild southern hairy-nosed wombats (*Lasiorhinus latifrons*). Fecal androgen concentrations for devils and spotted-tailed quolls were twice those measured in wombats (Hamilton *et al.* 2000), but considerably lower than those reported for a wide range of male eutherians, including other carnivores (e.g. black-footed ferret, hyena: Brown 1997; Dloniak *et al.* 2004).

5.5.2 Pattern of plasma and fecal androgens

In male marsupials, rising androgens generally stimulate spermatogenesis, and the development of male accessory organs (Tyndale-Biscoe and Renfree 1987); however, elevated concentrations are not necessarily required for maintenance of spermatogenesis, and may also result from intra-specific aggression and sexual encounters during the

breeding season (Inns 1982; Bryant 1986; Gemmell *et al.* 1986; Woolley 1990b). For devils and spotted-tailed quolls, the timing of the increase in fecal androgens began in advance of the main breeding period. This is the typical pattern in most seasonally breeding male marsupials and other mammals - a rise in plasma androgens generally precedes the onset of female estrus activity (Lincoln 1981; Tyndale-Biscoe and Renfree 1987). In our devil population, ovarian steroid monitoring of the females revealed that they entered estrus in late January (Hesterman *et al.* 2008a), up to six weeks earlier than previously determined from birth data (Green 1967; Guiler 1970a; Hughes 1982). Estrus in spotted-tailed quolls began in mid-May (Hesterman *et al.* 2008b), around a month sooner than other reports for the species (Fleay 1940; Green and Scarborough 1990; Belcher 2003). In male devils and spotted-tailed quolls, plasma and fecal androgen concentrations peaked early in the mating season. This is similar to the pattern of plasma androgens in *Sminthopsis* (McDonald *et al.* 1981; Woolley 1990b), whereas in other dasyurids, this peak tends to occur during the main mating period (Bradley *et al.* 1980; McDonald *et al.* 1981; Wilson and Bourne 1984; Bryant 1986; Bradley 1987; Schmitt *et al.* 1989; Millis *et al.* 1999). Semelparous dasyurids such as *Antechinus* and the red-tailed phascogale (*P. calura*) present an exception: in those species, androgen concentrations continue to increase during the breeding season, resulting in stress related post-mating mortality of males (Bradley *et al.* 1980; McDonald *et al.* 1981; Bradley 1987). The major difference in the androgen patterns of the devil and the spotted-tailed quoll and all other dasyurids that have been studied was the sustained duration of the seasonal elevation in androgens.

Female devils and spotted-tailed quolls are facultatively polyestrous (Hinds 1989), like most iteroparous dasyurids (Tyndale-Biscoe and Renfree 1987), and undergo a second cycle if they experience breeding failure (Hesterman *et al.* 2008a; 2008b). In the present study estrus activity in captive devils and spotted-tailed quolls continued over approximately four and a half to five months, respectively (Hesterman *et al.* 2008a; 2008b). It is likely that the long period of elevated androgen concentrations ensures males remain behaviourally and physiologically capable of procuring/fertilising females that undergo later estrous cycles. In *Sminthopsis*, breeding also occurs over an extended period (6 – 9 months) because females rear two litters a year (Morton 1978; Woolley

1990a), however, androgen concentrations in males are not sustained for this duration (McDonald *et al.* 1981; Woolley 1990b).

The annual patterns of androgens in the spotted-tailed quoll and the devil are more comparable with those reported for the brush-tailed possum and northern brown bandicoot (Gemmell *et al.* 1985; Gemmell *et al.* 1986), which are not strictly seasonal breeders. Dasyurids typically exhibit a restricted, well-regulated annual breeding period (Lee *et al.* 1982; McAllan 2003). In fact, devils were previously considered strict, highly synchronised breeders because mating and births are reported to occur over a brief period of several weeks in March and April (Green 1967; Guiler 1970a; Hughes 1982). By comparison, spotted tailed quolls usually breed in June/July (Fleay 1940; Green 1967; Belcher 2003) and mating is not tightly synchronised within populations (Belcher 2003). Our research (Hesterman *et al.* 2008a; 2008b; this study) has revealed that in captive devils and spotted-tailed quolls the mating period can extend for three – four months, and there is some evidence that implies that the breeding season may also be protracted in free-ranging populations. Hughes (1982) determined that in devils spermatogenesis can continue at least until August, and several incidences of births have been noted for that month (Green 1967; Guiler 1970a). More recently there is a report that suggests spotted-tailed quolls can produce young as late as September (Körtner 2006). Further examination of seasonality in the remaining wild populations is required to determine the limits of the breeding season in these large carnivorous marsupials.

In conclusion, the present study has increased our understanding of dasyurid reproductive biology and breeding strategies by characterizing the annual pattern of androgens in male Tasmanian devils and spotted-tailed quolls, and further demonstrated the effectiveness of fecal hormone measurement as a non-invasive technique for monitoring gonadal steroids in marsupials. Longitudinal profiling of male devils and quolls revealed comparatively sustained elevations in fecal androgen concentrations to other seasonally breeding marsupials. Combined with scattered observations from wild devil and spotted-tailed quoll populations (Guiler 1970a; Hughes 1982; Körtner 2006), these results suggest a period of extended or continual spermatogenesis in the two largest members of the Dasyuridae. This information has implications for future captive breeding and research into assisted reproductive techniques in these threatened species.

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CHAPTER 6
FLEXIBILITY IN REPRODUCTIVE SEASONALITY AND SYNCHRONY IN THE TWO LARGEST MARSUPIAL CARNIVORES

6.1 Introduction

A range of ecological factors operate selectively to ensure reproductive activity occurs during the optimal period that will result in successful rearing of young. Depending on their habitat, animals may experience an optimal season that is annually fixed, unpredictable or continual: Mammals in temperate regions usually exhibit a well-defined breeding period because of the predictability and progression of seasons. By contrast, in arid regions the optimal period is often unpredictable so breeding may occur at any time; while in the tropics, there is considerably less environmental variation permitting reproduction to continue throughout the year (Sadlier 1969; Bronson 1985). Regardless of their environment, all mammals are united in their need to produce young at an optimal time of year to ensure survival, and this ultimate factor relates to the availability of food (Bronson 1989). Breeding within this parameter confers advantages during susceptible life events such as late lactation, birth and/or weaning of young, and varies between species. The major influences on the timing of breeding are the lengths of gestation and lactation, because these are the periods when female and offspring are most vulnerable to climatic and environmental effects (Sadlier 1969). Gestation length tends to be a fixed character in mammals: the time from birth to weaning, therefore, determines the appropriate timing of conception, although reproductive mechanisms such as delayed implantation and embryonic diapause can alter gestation length and reduce the time between conceptions (Sadlier 1969; Hinds and Loudon 1997). Other features of the female cycle will also affect the limits of the breeding season; for example, whether a species is monoestrous or polyoestrous, and whether or not the females experience lactational anoestrous (Bronson 1989).

Timing of reproduction is also influenced by a host of proximate factors such as dietary and social cues, as well as photoperiod, temperature, rainfall and humidity (Sadlier

1969; Bronson 1985; Tyndale-Biscoe and Renfree 1987; McAllan 2003). Food intake is considered to be the most fundamental of all these ambient factors (Bronson 1985; Lee and Cockburn 1985), which is not unexpected, given the ultimate importance of this resource to overall well-being and survival. Bronson (1985) proposes that studying the bioenergetic control of reproduction is the most appropriate approach for understanding the evolutionary forces that have shaped mammalian breeding success.

Phylogenetic adaptations of different species present additional biological effects on breeding, particularly those relating to diet and body size (Bronson 1985; Lee and Cockburn 1985). Dietary specialisation (*e.g.* herbivory, insectivory, carnivory) has a major influence on shaping reproductive patterns of mammals and synchronising breeding events. Superimposed on this are allometric constraints associated with body size, which include different energetic requirements associated with growth, metabolism and reproduction (Bronson 1985; Cockburn and Johnson 1988).

Marsupials are a useful group to inform us about breeding processes in mammals because in several key features they present a dichotomy to eutherians. These include an extended duration of maternal investment, slower postnatal growth, reduced metabolic rates and conservative body size (Lee and Cockburn 1985; Cockburn and Johnson 1988; Tyndale-Biscoe 2005). Energetic investment in reproduction is similar between the two groups, but in marsupials the major difference is that the major energetic costs of breeding are concentrated during the period of late lactation, while they are but evenly distributed throughout pregnancy and lactation in eutherians (Russell 1982; Lee and Cockburn 1985; Tyndale-Biscoe 2005). Long lactation means that reproduction occupies a large proportion of the year for female marsupials, and so mating and birth usually occur well in advance of the optimum period for weaning when food is abundant (Tyndale-Biscoe 2005). This pattern, in which conception is timed to occur during an inclement period of the year, is also found in eutherians with a lengthy reproductive cycle (*e.g. Ovis aries*) (Sadlier 1969).

The Dasyuridae, or carnivorous marsupials, are a particularly interesting group in which to study timing of reproduction. They are speciose and ecologically diverse, represented by more than 60 species from 10g insectivores (planigale) to 14 kg flesh-eating

carnivores (Tasmanian devil) found exclusively in Australia and New Guinea (Strahan 2005); they inhabit a range of vastly different climatic zones and habitats from arid areas, grasslands and woodlands, to tropical and temperate rainforest (Krajewski *et al.* 2000). In addition to the marsupial attributes outlined above, they have exceptionally short lifespans, epitomised by the evolution of semelparity in dasyurids such as *Antechinus* - renowned for the abrupt, post-mating mortality of males (reviewed in Dickman 1993; Cockburn 1997; Bradley 2003). Mortality is an important predictor of variation in mammalian life histories, and strongly correlated with body mass (Promislow and Harvey 1990). Seasonality and predictability of environment are also shown to be associated with life history patterns (Taggart *et al.* 1997). Accordingly, dasyurids are characterised by a diversity of reproductive strategies.

Six life history categories have been defined for dasyurids. These represent a continuum based on specific parameters including age at sexual maturity, seasonality and the frequency and duration of male and female reproductive effort (Lee *et al.* 1982). Strategy I and II species mature at 11 months of age, are ecologically monoestrous and have restricted breeding periods with (I) or without (II) male die-off. Strategy III species share the same features as Strategy II, except females are facultatively polyoestrous. Strategy IV and V species are also polyoestrous, but sexual maturity is attained earlier (6 - 8 months) and breeding occurs over an extended period. Aseasonal breeding is the only characteristic that distinguishes Strategy VI dasyurids from Strategy IV and V species.

Past studies have focused on the breeding ecology of smaller, insectivorous dasyurids including semelparous and iteroparous species (Cuttle 1982; Fanning 1982; Taylor *et al.* 1982; Woolley and Ahern 1983; Soderquist 1993b; Friend *et al.* 1997; Oakwood 2000). In particular, research has centred on physiological mechanisms and ecological influences of reproduction and life history in insectivorous, semelparous *Antechinus* species from mesic, temperate regions of south-eastern Australia and iteroparous *Sminthopsis* from the arid zone. No comparative research has been published on the two largest dasyurids, which are the only predominantly flesh-eating marsupials in Australia - the Tasmanian devil (*Sarcophilus harrisii*) and the spotted-tailed quoll (*Dasyurus maculatus*). The spotted-tailed quoll is the largest quoll and classified with the eastern

quoll (*D. viverrinus*) and the western quoll (*D. geoffroii*) as having a type III life-history strategy (Lee *et al.* 1982; Krajewski *et al.* 2000; McAllan 2003). A lack of information on features of the reproductive biology of the Tasmanian devil, such as age at sexual maturity and pattern of oestrous, has presented an ongoing problem for life history classification. As a result, the devil's life history strategy remains inferred or uncertain (Lee *et al.* 1982; Krajewski *et al.* 2000; McAllan 2003).

The objective of this study was to determine the parameters of the reproductive season and evaluate the timing of breeding of the Tasmanian devil and spotted-tailed quoll, two large-bodied, flesh-eating carnivorous marsupials, in a climatically predictable temperate environment. To address this aim, data were collected from devil and spotted-tailed quoll populations at two sites in north-eastern Tasmania that represent the variety of natural habitats for these species. First, it was necessary to establish the physiological and physical characteristics that define reproductive status through monitoring gonadal activity, as well as collecting morphometric and demographic information. This evaluation permitted parameters such as onset of sexual maturity, and the timing/duration of oestrous activity and spermatogenesis in the populations to be determined, so the limits of the breeding season could be established for both the female and the male of each species. This information is necessary to fill gaps in our understanding of these ecologically distinct species, and to better appreciate the diversity of life history strategies in dasyurid marsupials. Findings may further our knowledge of the ecological processes that influence the timing of reproduction in other mammalian species.

6.2 Materials and Methods

6.2.1 Study Sites

Field studies were conducted at two sites in Tasmania: 1) the Mersey/Meander (MM) district in the state's north, encompassing the northern escarpment of the Great Western Tiers (maximum elevation sea level to 1420m), Gog Range and Needle's Ridge to the north; and 2) the Freycinet Peninsula (FNP) on the northeast coast. The Mersey/Meander district includes a variety of habitats from dry sclerophyll, heathland and pasture to cool temperate rainforest, and habitat type varies with altitude. Annual

temperature ranges from –0.9 – 10.4°C in winter (July) and from 8.7 – 22.5 °C in summer (February); mean annual rainfall is 950 mm, increasing toward the north western aspect of the Tiers. The Freycinet Peninsula is predominantly dry sclerophyll forest, with some heathland, wet sclerophyll forest and pasture; mean annual temperature range ranges from 6.0 - 14 °C in mid-winter (July) and from 13.0 – 21.4°C in late summer (February); mean annual rainfall is 670 mm (Australian Government Bureau of Meteorology, http://www.bom.gov.au/climate/averages/tables/cw_092003.shtml).

6.2.2 Trapping and Assessment of Population Structure

Twenty five - 30 wire-cage carnivore traps baited with meat were set for six consecutive days study locations in the Meander/Mersey district each month from January to December 2001, with the exception of April when concurrent research on captive populations was underway. The Freycinet peninsula study site extended from Bicheno in the north to the southern part of the Peninsula (110 traps in 160 km^2). The peninsula was divided into four areas that were trapped typically for seven nights each June/July from 1999 to 2004. In addition, the two central areas (North and South) were trapped in January, April and November in 2000 – 2002. Data on devils from 1999 – 2001 are included; while spotted-tailed quoll data includes that collected until 2004.

Traps were cleared daily and reset. Animals were transferred to a handling bag and restrained unanaesthetised while data on morphometrics, age, sex and reproductive status were recorded and samples collected. Trapped animals were individually marked with a unique number tattooed inside the ear, and released at the site of capture.

Body mass was measured using Salter scales (accuracy to 100 g). Head width and urogenital sinus or scrotal width and length were measured using vernier calipers (accuracy to 0.1 mm) Scrotal width was taken with the stalk extended, and lengths of the left and right portion of the scrotum were measured individually. Devils and spotted-tailed quolls were aged using a combination of body mass, head width, molar tooth eruption, canine over-eruption and canine and molar tooth wear, using protocols developed by Belcher (2003) (quolls), Pemberton (1990) and Jones (unpublished) (devils), respectively. Devils can be definitely aged to 2 years; accuracy declines with increasing age, because after this because there are individual and site differences in

tooth wear and canine over-eruption. The majority of devils in the Freycinet study were of known age because nearly all individuals in that population had been trapped and individually earmarked for identification in previous years.

Pouches were examined to determine female reproductive status (anoestrous, oestrous, presence of pouch young (PY), lactation). Breeding condition of females was determined following characteristic physical changes in pouch appearance during oestrous (size, colour and secretions) detailed in Hesterman *et al.* (2008a) (see Chapter 2). Pouch young were counted, sexed, and measured using vernier callipers to record head width (HW), and crown-rump length (CRL). Stage of PY development was also noted, including presence of pelage, definition of the eyes and ears, and formation of the mouth and lips (after Guiler 1970). Birth date was estimated to the nearest week by ageing PY from growth curves constructed from known age captive litters (devils: Appendix C; quolls: Collins *et al.* 1992). For lactating females, the number and size of teats (length x width to nearest 0.1 mm) were also recorded.

6.2.3 Sample Collection and Analyses

Plasma and Faecal Sex Steroids

Blood samples were collected within five minutes of removal of the animal from the trap. 75-150 µL blood was collected from the peripheral ear by venipuncture with a lancet and heparinised capillary tube. Samples were stored on ice in the field and centrifuged later that day; recovered plasma was stored frozen (-20°C) until radio-immunoassay (RIA). Plasma was extracted and analysed for testosterone or progesterone, following methods detailed in Hesterman and Jones *et al.* 2008a and Hesterman and Jones (2008) (see Chapters 2, 4). Intra-assay and inter-assay coefficients of variation were 8.4 % and 9.3 % for testosterone, and 9.5 % and 10.2 % for progesterone. Assay sensitivity, measured as the least detectable dose of steroid, was 0.06 ng/ml for androgens and 0.09 ng/ml for progesterone.

Faecal samples were removed from inside the trap and placed in a ziplock bag on ice, then stored at -20°C for later processing. Where several scats were present the visibly freshest was selected. Samples were lyophilised and screened to remove fur, bones and other fibrous or undigested matter, and refrozen (-20°C) until extraction and assay.

Male samples were analysed for testosterone by radioimmunoassay, and female samples were analysed for 20α-OH-pregnanes (pregnanediol), 20-oxo-pregnanes, and total oestrogens by enzyme-immunoassay (EIA) (see Hesterman and Jones 2008, and Hesterman *et al.* 2008a for details) (Chapters 5 and 2, respectively). Intra-assay and inter-assay variation were < 10 % and 15 % respectively, for all faecal steroids. Assay sensitivity was 2 ng/g for testosterone and 2 ng/g for progestagens and total oestrogens.

Urogenital (vaginal) smears

Urogenital smears were obtained from the posterior vaginal sinus by introduction of a small cotton swab through a glass speculum. Smears were air-dried and stored, then later stained. Cell populations were examined at x 40 magnification to assess the relative proportion of epithelials, parabasals and leucocytes per 100 cells/slide.

Testicular biopsies

Testicular aspirates were collected from adult (> 2 yrs) Tasmanian devils at the Meander/Mersey study site during September – December, by fine needle biopsy (FNAB). The scrotum was swabbed with alcohol, and a 23 gauge needle inserted at a shallow angle into the testicle to avoid the vascular epididymis. The resulting droplets were smeared onto a clean microscope slide and air dried for later processing. Testicular smears were stained with Diff-Quik (Laboratory Aids, Narabeen, Australia) and examined under a microscope at x 40 magnification for cytological changes associated with spermatogenesis.

Gross Anatomy and Histology

Data were collected from recently road killed carcases, or animals that were euthanised by veterinarians as a result of injuries. The majority of animals were collected from the northern end of the main highway that bisects Tasmania north to south (A1), and from roads near the two field study sites.

Postmortem animals were aged, and bodies were weighed and measured as described for live captures. Male and female reproductive organs or tracts were dissected free of connective tissue, measured with vernier callipers to 0.1 mm and weighed to the nearest 0.1 g. For males, the scrotum was removed at the distal end of the stalk and weighed, before the testes and epididymides were extracted for further measurements. Length and

width of each testis was taken and the testes and epididymides were weighed individually The prostate was removed and the length and maximal width measured, then weighed. For females, the width and length of the urogenital sinus, cervix (= uterine neck, Pearson & De Bavay 1953), uterii and ovaries were measured. Fresh samples harvested immediately post-mortem were fixed in Bouins's solution or 10% buffered formalin. Testes and epididymides were embedded in paraffin wax and sectioned at 3 µm; serial sections were stained with haematoxylin and eosin for histological examination.

6.2.4 Statistical Analyses

All data are presented as the mean ± SE, except where indicated otherwise. The year was divided into four seasons: December-February (summer), March - May (autumn), June - August (winter) and September - November (spring). Student's unpaired t test or analyses of variance (ANOVA) were used to compare hormone concentrations and mass or size of reproductive tracts or accessory organs among months or seasons ($P < 0.05$). Tukey's post-hoc comparison was used to determine significant differences between groups. Chi-square was applied to test for differences between age classes and number of young produced in the population. Statistical analyses were performed using SPSS (SPSS Inc. 1998, Chicago IL), Version 13 package.

6.3 Results

6.3.1 Study Animals

213 Tasmanian devils (90 males; 123 females) were trapped at MM, and 306 devils (145 males; 161 females) at FNP. Population structure varied, but all age classes were represented at both sites from recently weaned juveniles (~ 9 months old) to adults up to six years of age. For devils, mean bodymass and range were similar for males at both study sites, averaging around 7.5 kg (MM = 8.0 ± 0.24 kg, range 1.7 – 13.2 kg; FNP = 7.4 ± 0.16 kg, range 2.7 – 14.5 kg) ($t_{(574)} = -0.88$, $P = 0.382$). Male devils were significantly heavier than females ($t_{(932)} = 11.53$, $P < 0.01$). Females weighed between 6.0 – 6.5 kg (MM = 6.0 ± 0.14 kg, range 1.3 – 10.2 kg; FNP = 6.7 ± 0.13 kg, range 1.9 – 12.8 kg), and were significantly heavier at FNP ($t_{(356)} = 3.37$, $P < 0.01$).

Thirty-four spotted-tailed quolls (18 males; 16 females) were captured at MM, and 18 individuals (11 males, 7 females) at FNP. The animals ranged in age from a dependent young (estimated at 6 months of age) to adults of approximately four years old. Quoll bodymass for each sex was comparable between study sites, but males were significantly heavier than females ($t_{(52)} = -6.31$, $P < 0.001$). Males weighed ~3.0 kg (MM = 3.2 ± 0.19, range 1.8 – 4.4; FNP 2.8 ± 0.26, range 1.0 – 4.3) ($t_{(29)} = 1.35$, $P = 0.189$), while females were only ~1.8 kg (MM = 1.8 ± 0.10; range 1.1 – 2.7; FNP = 1.7 ± 0.11; 1.2 – 2.0) ($t_{(21)} = -0.66$, $P = 0.948$).

Postmortem data was obtained from 110 Tasmanian devils (59 males; 51 females), and 53 spotted-tailed quolls (39 males; 14 females), representing all age groups for both species.

6.3.2 Female Reproduction

Physiological and Physical Characteristics of Breeding Status

General

There were differences in the concentrations of plasma and faecal sex steroids, and the size, mass and appearance of the reproductive tract, associated with pouch condition in female devils and spotted-tailed quolls. There was a significant difference in plasma progesterone according to pouch condition for devils ($P < 0.001$) (Figure 1). Quolls with an oestrous pouch had higher concentrations of plasma progesterone than other adults, (OE vs PY and LACT $t_{(24)} = -2.197$; $P = 0.03$) (Table 1). There was a significant positive correlation between plasma progesterone and fecal pregnanediol for both species (*devils:* $y = 1.0333x - 2.558$, $R^2 = 0.975$; $P < 0.001$; $n = 22$; Pearson = 0.987 at 0.01 level; *quolls:* $y = 1.1307x - 2.124$, $R^2 = 0.747$; $P < 0.001$; $n = 11$; Pearson = 0.864 at 0.01 level). Compared to plasma progesterone, faecal progestagen concentrations were more variable for devils (Figure 2a) and quolls (Table 1), but showed a similar trend with pouch condition (STQ: $F_{3,15} = 3.215$; $P > 0.05$; TD: $F_{3,23} = 0.987$; $P > 0.05$). Fecal oestrogen concentrations were most elevated in female devils (Figure 2b) and quolls (Table 1) with an oestrous pouch appearance (STQ: $F_{3,18} = 1.331$; $P > 0.05$; TD: $F_{3,25} = 0.691$; $P > 0.05$).

Table 1

Plasma and faecal sex steroid concentrations in free-ranging female spotted-tailed quolls (*D. maculatus*). IMM = immature; AO = anoestrous; OE = oestrous; PY = pouch young; LACT = lactating

Pouch Condition / Breeding Status	Plasma Progesterone (ng/ml)	Faecal Pregnanediol (ng/g)	Faecal Oestrogens (ng/g)
IMM	0.05 ± 0 02 (2)	-	-
AO	0.07 (1)	136.8 ± 11.46 (3)	3.3 ± 0.18 (3)
OE	3.06 ± 2.87 (5)	4353.4 ± 663.71 (5)	22.4 ± 23.14 (5)
PY	0.33 ± 0.28 (6)	2454.6 ± 444.71 (5)	45.6 ± 49.42 (5)
LACT	0.11 ± 0.07 (15)	286.7 ± 45.55(8)	11.6 ± 10.58 (8)

Immature Animals

Immature (IMM) female devils and spotted-tailed quolls had a characteristically small, undeveloped pouch. Average body mass for devils with an immature pouch was around 5.0 kg (FNP: 5.0 kg ± 0.10, n = 100; MM: 4.7 kg ± 0.16, n = 50), and most animals were estimated to be less than two years of age. All quolls with an immature pouch were estimated as less as one year old and had an average body mass of 1.2 ± 0.07 (FNP n = 1; MM n = 3). For immature devils, average plasma progesterone and faecal pregnanediol (PgD) and total oestrogens were lower compared to mature individuals (Figures 1 and 2). Urogenital smears contained a high proportion of parabasals (~ 70 %) and fewer cornified epithelial cells (~ 10 %) than samples from sexually mature females. Only three immature quolls were bled, but similarly to devils, plasma progesterone concentrations were low (0.05 ± 0.02 ng/ml) compared to breeding females.

For devils, there were also significant differences in the size and mass of the vaginal complex in relation to pouch condition (ovary length, width and mass $P < 0.01$; uterus length, width and mass $P < 0.01$; cervix width $P = 0.01$, cervix length $P = 0.01$). A similar pattern of change was apparent for spotted-tailed quoll, but insufficient data were available to perform statistical analyses. Postmortem data showed that devils with an undeveloped pouch area had significantly smaller reproductive tracts (Figure 3) compared to all other females. In the one immature quoll examined, the reproductive

tract and associated organs were substantially smaller (uterii = 0.06 g, 4.1mm length x 3.3mm width; ovaries = 0.02 g, 4.1 mm length x 2.8 mm width) than the average mass and dimensions for mature female quolls (uterii = 0.83 ± 0.31 g, 14.3 ± 5.06 mm length x 8.8 ± 1.09 mm width; ovaries = 0.16 ± 0.03 g, 7.5 ± 0.51 mm length x 5.7 ± 0.30 mm width). No follicles were visible on the ovarian surface of any animals with an immature pouch.

Mature Animals

Adult females (2 yrs +) trapped outside of the main breeding season (TD: Dec or Jan; STQ: Jan – May) usually had an enlarged but inactive pouch (pale, no PY or enlarged/lactating teats), indicating an anoestrous state (AO). Anoestrous devils and quolls had lower average concentrations of plasma progesterone (TD: Figure 1; STQ: 0.04 ng/ml ± 0.02; n = 3) than breeding individuals (TD: Figures 1 and 2; STQ: 0.73 ng/ml ± 0.56; n = 26). For anoestrous quolls, average faecal sex steroids were also lower than in breeding adults (Table 1). For devils with an anoestrous pouch, reproductive tracts were not significantly larger or heavier than those of immature individuals (Figure 2), and the ovarian epithelium was smooth with no visible follicles.

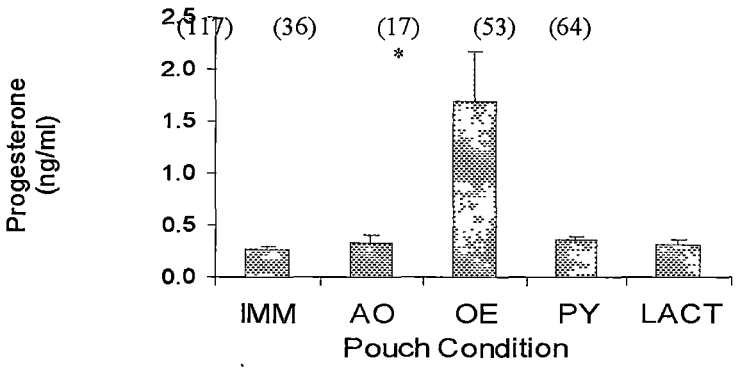


Figure 1: Plasma progesterone concentrations (ng/ml) associated with pouch condition in free-ranging female Tasmanian devils. IMM = immature; AO = anoestrous; OE = oestrous; PY = pouch young; LACT = lactating. Values are mean ± SEM; sample sizes indicated in brackets. Asterix (*) indicates significance $P < 0.01$.

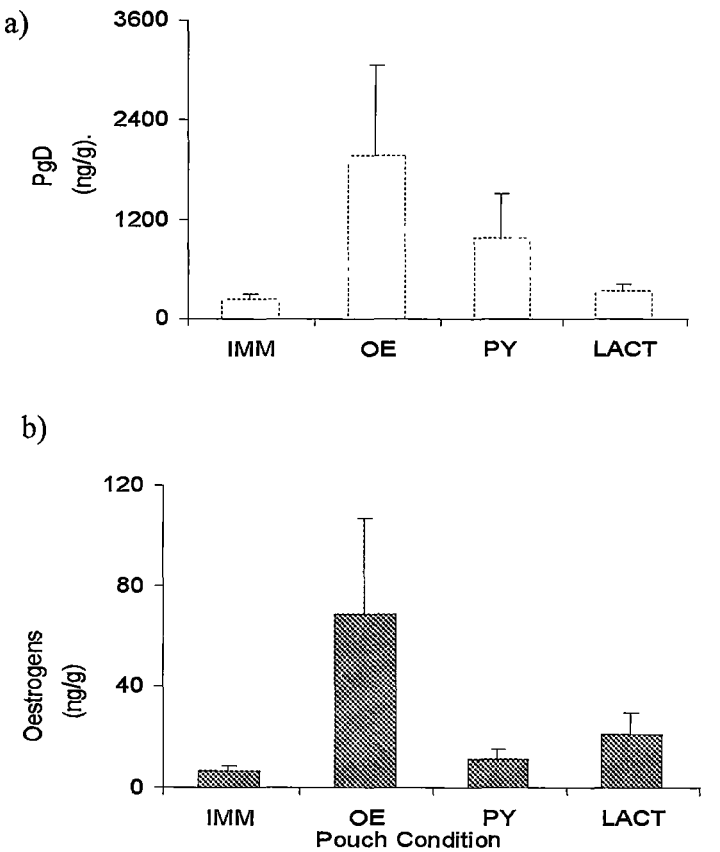


Figure 2:: Faecal a) pregnanediol (PgD) and b) total oestrogen concentrations (ng/g) associated with pouch condition in free-ranging female Tasmanian devils. IMM = immature; AO = anoestrous; OE = oestrous; PY = pouch young; LACT = lactating. Values are mean ± SEM ; n = six for each group.

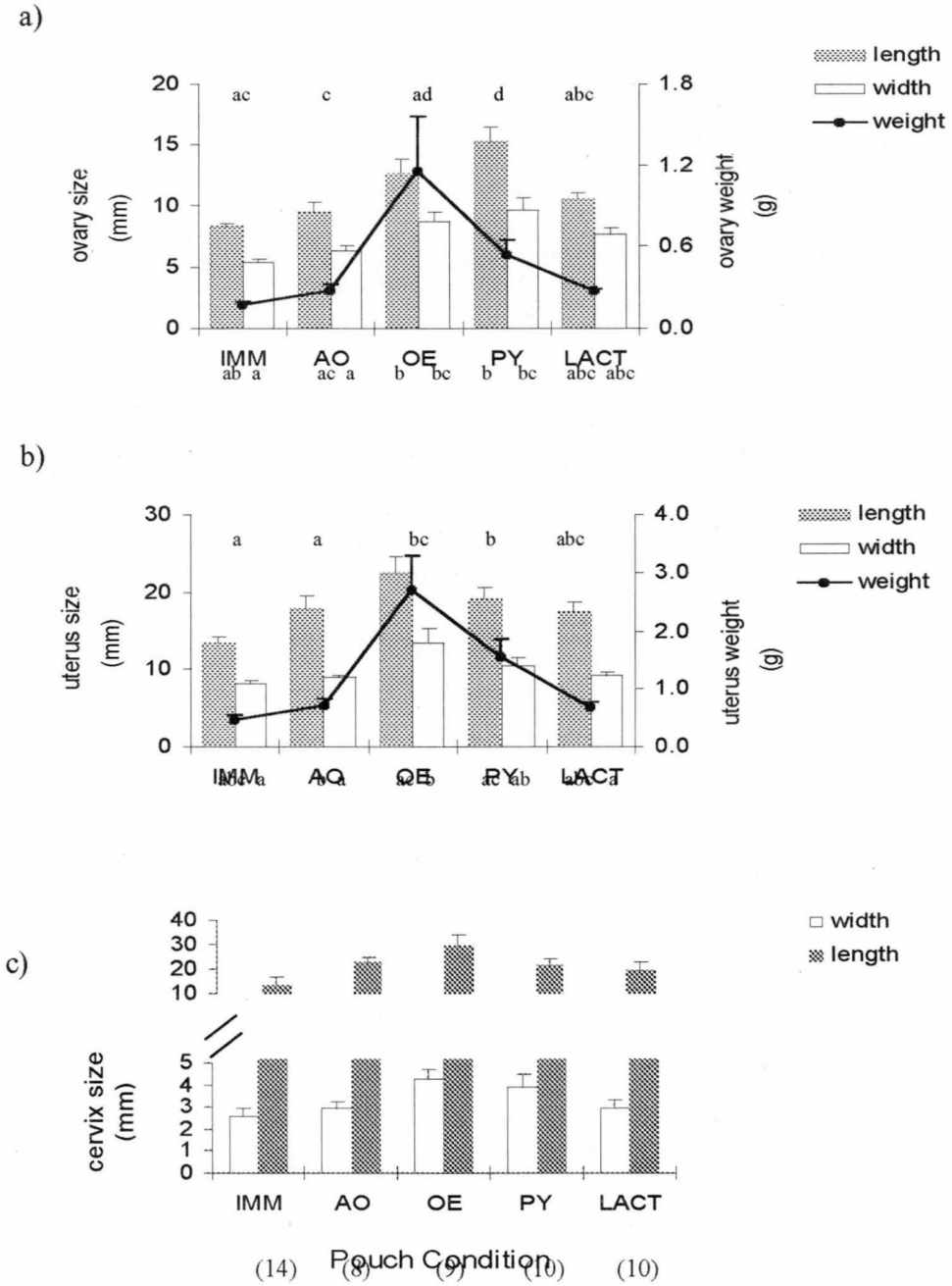


Figure 3:

Changes in a) ovary and b) uterus size (mm) and mass (g), and c) cervix size (mm) in Tasmanian devils, associated with pouch condition. Values are mean ± SEM; sample sizes indicated in brackets below c). Note break in y-axis in bottom graph. X. IMM = immature; AO = anoestrous; OE = oestrous; PY = pouch young; LACT = lactating. Values with different letters indicate significant differences between groups ($P < 0.05$); indicated below bars for width and length, and above for mass.

During the breeding season, the pouch of adult devils and spotted-tailed quolls developed and showed signs of glandular activity – either the presence of an oily, red exudate and swelling of the area (oestrus), or marked deepening and the presence of thin, clear fluid (post-ovulatory). These characteristics classified an oestrous pouch (OE). For devils, average concentrations of plasma progesterone for females with an oestrous pouch were variable, but significantly higher than other groups (Figure 1; $F_{(4, 281)} = 20.14$; $P < 0.01$), and average concentrations of faecal oestrogens and progestagens showed a similar trend (Table 1; PgD: $F_{(3, 26)} = 0.90$, oestrogens: $F_{(3, 25)} = 0.96$; $P > 0.05$). Similarly, for quolls plasma and faecal sex steroids were also variable in individuals with an oestrous pouch; however, all three hormones were in greater concentrations compared to other reproductive conditions (plasma progesterone: 3.06 ± 0.29 ng/ml $F_{(2, 23)} = 2.33$; $P > 0.05$; for faecal steroid concentrations see Table 1; PgD: $F_{(2, 15)} = 1.38$; $P > 0.05$, oestrogens: $F_{(2, 15)} = 1.87$; $P > 0.05$).

Urogenital smears of oestrous females contained less parabasals and a higher percentage of epithelial cells (~ 45 %) than immature or anoestrous females. Female devils with an oestrous pouch had significantly larger reproductive organs (*ovary length*: $F_{(4, 42)} = 11.51$, $P < 0.05$, *ovary width*: $F_{(4, 42)} = 7.13$; $P < 0.05$; *uterus length*: $F_{(4, 42)} = 5.73$, $P < 0.05$; *uterus width*: $F_{(4, 41)} = 5.56$, $P < 0.05$), heavier ovaries ($F_{(4, 42)} = 5.09$, $P < 0.05$) and uterii ($F_{(4, 41)} = 12.35$, $P < 0.05$) than other groups (Figure 2a, b). The size of the uterine cervix followed a similar pattern (*length:* $F_{(4, 22)} = 2.24$, $P > 0.05$; *width:* $F_{(4, 27)} = 2.44$, $P > 0.05$) (Fig 3c). Gross appearance of the ovaries was transformed in devils with an oestrous pouch - distorted by >150 corpora lutea (1 – 4mm diameter), which lent a 'raspberry-like' appearance to the ovary. One female also showed marked swelling of the lateral vaginae (*w* x *h* = 18 0 mm x 9.0 mm).

Although few adult female quoll were available for each group (AO n = 3; OE n = 2, PY n = 2; LACT n= 6), a similar trend in gross reproductive anatomy was apparent to that observed for devils. In the two females with oestrous pouches uterii size (female A = length x width = 36.8 x 21 mm; female B = 24.9 x 15.4 mm) and mass (female A = 0.94 g; female B = 3.5 g) was greater compared to anoestrous (see above) or lactating quolls (average uterii *l* x *w* = 11.7 ± 0.71 mm x 7.0 ± 0.48 mm; *mass* = 0.38 ± 0.04 g). Quolls with an oestrous pouch also considerably larger, heavier ovaries (female A = 36.8 mm *l*

x 21.0 mm *w*, mass 0.29 g; female B = 24.9 mm *l* x 15.4 mm *w*, mass 0.23 g) than either anoestrous or lactating individuals. In one of the females (A) multiple follicles (< 2 mm diameter) and corpora lutea extruded from the surface of both ovaries. A urogenital smear taken from this animal contained > 90% superficial epithelial cells. The lateral vaginae was visibly enlarged in both females with an oestrous pouch (*w* x *h* = 6.4 mm x 3.6 mm and 18.8 x 9.5 mm).

Devils trapped either with pouch young (PY) or during lactation (LACT) had lower and less variable average plasma and faecal sex steroid concentrations than females with an oestrous pouch (Figure 1a, b; Table 1). Quolls carrying pouch young had higher values and individual variation for plasma progesterone (0.33 ± 0.28, n = 6) than lactating females (0.11 ± .0.07, n = 16) ($t_{(19)}$ = 1.11, P > 0.05). Faecal steroid concentrations similarly revealed high individual variation for quolls with PY (Table 1). For devils with pouched or denned young, the average mass of the reproductive organs was markedly lower than that of females with an oestrous pouch (Figure 2a,b), and the average uterus mass and dimensions were similar to anoestrous females (Figure 2c). The ovaries of female devils with young were still relatively enlarged, but no follicles were evident at the surface. The lateral vaginae of two of these devils were greatly enlarged (*w* x *h* = ~16.0 mm x ~4.0 mm). For quolls with PY, ovary dimensions (female C = *l* x *w* 9.2 x 5.85 mm; female D = 9.9 x 7.5 mm) and total ovarian mass (female C = 0.23 g; female D = 0.35 g) was also heavier than those observed in anoestrous or lactating females (see above). Both quolls had small young (CRL ≤ 18.5 mm, estimated ≤ 3wks old) and their ovaries were of similar size and mass to those of oestrous quolls. Small follicles (~1.0 mm diameter) were still visible on the epithelial surface of the ovaries of Female C. The uterii of females with PY were enlarged (*l* x *w* female C = 14.6 x 18.4 mm; female D = 14.4 x 11.3 mm) compared to anoestrous and lactating females, but only approximately half the size of uterii in oestrous females.

Breeding Pattern

Tasmanian devils

The majority of females aged from 2 – 4 years produced young, and different age groups contributed similarly to breeding within the population (Table 2). Overall success was limited to approximately 45 % of the total population; however, this figure was skewed

by the over-representation of one year old, immature females (FNP = 54 %; MM = 44 %). The percentage of individuals that produced young in the mature population (aged 2 + years) was considered more representative of breeding success, and ranged from ~ 70 – 90 % (Table 2). The median number of young produced at FNP and MM was similar, with most dams producing four young, being the maximum number of offspring that can be accommodated on the teats (Appendix C).

The percentage of females trapped with PY or LACT did not vary by year or location, but variation in the ages of breeding females was apparent (Table 2). Few devils produced young in their first year (~ 7 % of age class), and success was also low among devils older than four years (< 15% of age class), with none producing young after 5 years of age. Combined site data for one year old devils showed that females with young had significantly heavier body mass (6.8 ± 0.44 kg) than those that did not breed (5.3 ± 0.12 kg) ($t(91) = 4.122$, $P < 0.001$). Successful females aged 2+ years were not significantly heavier than those that did not breed (MM: breeding 7.0 ± 0.13 kg, non-breeding 6.5 ± 0.20 kg; $t(81) = 1.971$, $P = 0.05$; FNP: breeding 8.2 ± 0.13 kg, non-breeding 8.0 ± 0.71 kg; $t(114) = 0.464$, $P = 0.06$). Based on subsequent recaptures of individual dams, losses of young during lactation were uncommon. Three females with pouch young (2 FNP; 1 MM) recaptured 3 – 6 months later had replaced earlier litters.

Table 2

Female Tasmanian devils captured breeding (i.e. with pouch young or lactating) at Freycinet NP (FNP 1999 – 2001) and Meander district (MM 2001). Number of females trapped by age and study year; total number of individuals in brackets.

Age (yrs)	FNP 1999	FNP 2000	FNP 2001	MM 2001	% Total Age Group Breeding
1	2 (27)	5 (50)	2 (46)	3 (54)	6.8
2	16 (19)	18 (20)	15 (22)	19 (23)	81.0
3	5 (7)	11 (11)	8 (11)	16 (20)	81.6
4	1 (1)	5 (5)	4 (4)	17 (20)	90.0
5	1 (1)	1 (1)	1 (1)	0 (8)	14.3
6	0	0 (1)	0	0	0
% Total Population Breeding	43.6	44.2	35.3	44.0	
Aged 2 yrs + Breeding	78.6	92.1	68.3	77.5	

Spotted-tailed quolls

Most female quolls captured at MM or FNP were either carrying PY or lactating (Table 3). Females produced young in all age groups (from 1 – 4 years old), and nearly every individual aged 2 yrs + produced young (16 of 17 females). Only two first year old females were captured after reaching 12 months of age; one of these had young (Table 3), and a vaginal smear confirmed the other to be in oestrus (99% cornified epithelial cells). Most dams had a full complement of six young (median = 6). Only two females with young were re-trapped later in the same season (MM); both were rearing that same litter.

Table 3

Female Spotted-tailed quolls captured breeding at Freycinet (FNP 1999 - 2004) or Meander district (MM 2001 Number of females trapped by age and study year; total number of individuals in brackets.

	No. Females with Young		
Age (yrs)	MM 2001	FNP 1999 - 2004	Total
1	1 (4)	0 (1)	1 (5)
2	7 (7)	1 (1)	8 (8)
3	4 (4)	1 (2)	5 (6)
4	1 (1)	2 (2)	3 (3)
Total Litters	13	4	
Breeding Females	82%	88%	

Timing of Breeding Events

Tasmanian devils

Based on physiological data, oestrous activity occurred from 1 January – 21 May; births were recorded mid-January and mid-June (MM: weeks 2 – 24; FNP weeks 5 - 24) (Figure 4, inset). There was no significant variation in the mean week of birth between study sites (MM = 10.1 ± 0.88, FNP = 11.4 ± 0.33; $t_{(138)} = 1.63$, $P = 0.11$) or in different years at FNP (1999 – 2001; $F_{(2,88)} = 0.11$, $P = 0.90$). Births were distributed throughout the first six months of the year, although the majority occurred during the austral autumn (X^2 $P < 0.01$; df = 3; Figure 4). Approximately half (56%) of all litters were born during March (MM n = 11/22; FNP n = 57/92). The age of the dam had a highly significant effect on the week that they gave birth ($F_{(4,110)} = 4.648$; $P < 0.01$), with older females producing young earlier in the year. One year old females bred approximately one

month later (week 15.8 ± 2.0) than all other age groups (week 10.2 ± 0.3) ($P < 0.01$). Litters were produced as late as June (weeks 22 – 24) by females in most age groups (1, 2 and 4 yr old females).

Devils usually deposited their litters in a den from late June (FNP) to mid-August (MM) when the young were ~ 4 months old. Continued nursing by the young resulted in a pouch containing enlarged, lactating teats and a swollen udder. Lactation after pouch exit continued until late January/mid-February. Weaning occurred from December to late February with females undergoing lactational regression having characteristically hardened, lumpy udders with enlarged teats. A small number of dams were still lactating in June/July (n = 4), but the age of their young was unknown.

Spotted-tailed quolls

Oestrous activity was evident in quolls from 6 June - 20 August. Nearly all births were recorded between mid-June and the end of July (weeks 20 – 31; n = 5 litters) during the austral winter. At MM, one litter was produced during spring, in October (week 41). Lactating females without young were trapped between the end of September and end of January; regressed udders indicated that all litters were fully weaned by the end of February (n = 12).

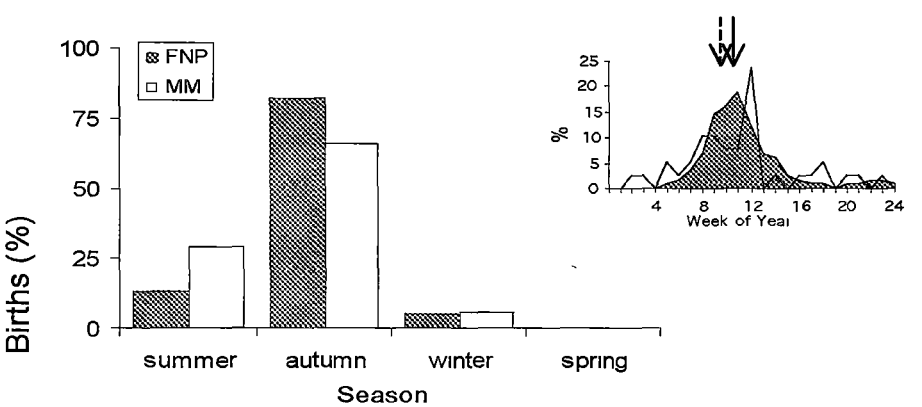


Figure 4:

Seasonality of births (%) in Tasmanian devils at Freycinet (FNP 1999 – 2001; n = 92 litters) and Meander district (MM 2001; n = 22 litters). Inset shows annual distribution of those births by week of year; arrows indicate mean birth week for each population (solid arrow = FNP, dashed arrow = MM).

6.3.3 Male Reproduction

Physiological and Physical Characteristics o f Breeding Status

Tasmanian devils

Plasma androgen concentrations for adult male Tasmanian devils were similar between sites ($t_{(221)}$ = 0.40, P > 0.05), and averaged 0.57 ± 0.05 ng/ml. Fecal androgen concentrations (MM only) averaged 20.11 ± 1.12 ng/g. There was a significant positive correlation between plasma and fecal androgens (y = 29.995x + 10.019, R^2= 0.936; P < 0.001; n = 40). Data were pooled to determine effects of month and season on plasma androgen concentrations. There was a significant seasonal change in plasma testosterone ($F_{3, 143}$ = 7.94; P < 0.001) and faecal androgen ($F_{3, 149}$ = 3.76; P < 0.05) concentrations (Figure 5a). In spring, prior to onset of the breeding season, males had significantly elevated androgen levels; and lowest concentrations were recorded during winter. Plasma data were also analysed by month to detect any underlying pattern of fluctuation (Figure 6). Concentrations were highest in November, and lowest in June/July ($F_{11, 135}$ = 3.85; P < 0.05).

There was a significant effect of season on body mass for male devils at MM (Table 4; P < 0.05), with heaviest mass recorded during winter/spring. Scrotal width increased during spring and peaked in spring/summer (FNP P < 0.05; MM P > 0.05); at FNP scrotal length also increased in spring (P < 0.05), but no change was apparent at MM (P > 0.05) (Table 4). Data from postmortem animals did not show any significant seasonal change in either bodymass ($F_{(3, 30)}$ = 1.30, P > 0.05) or scrotal size ($F_{(3,39)}$ *width* = 1.53; *length* = 0.91; P > 0.05). There was no corresponding change in testes mass or size (Tables 5 and 6) throughout the year (P > 0.05), or epididymal mass (Table 5). Similarly, no seasonal changes were apparent for prostate size (length and width) (Table 6; P > 0.05) or mass (P > 0.05, Figure 5b), although monthly data showed peaks in May and December (Figure 6).

Testicular biopsies and histology showed spermatogenesis occurred between November and August. Complete spermiogenesis occurred in these months, with spermatogonia, spermatocytes, spermatids observed in the seminiferous and epididymal tubules, and the presence of mature luminal spermatozoa. Variation was observed in the level of epithelial activity and numbers of Sertoli and germ cells present. In September and

October the epithelium was apparently depleted, fewer spermatogonia and Sertoli cells were evident and although some maturations stages were present the tubules were aspermic.

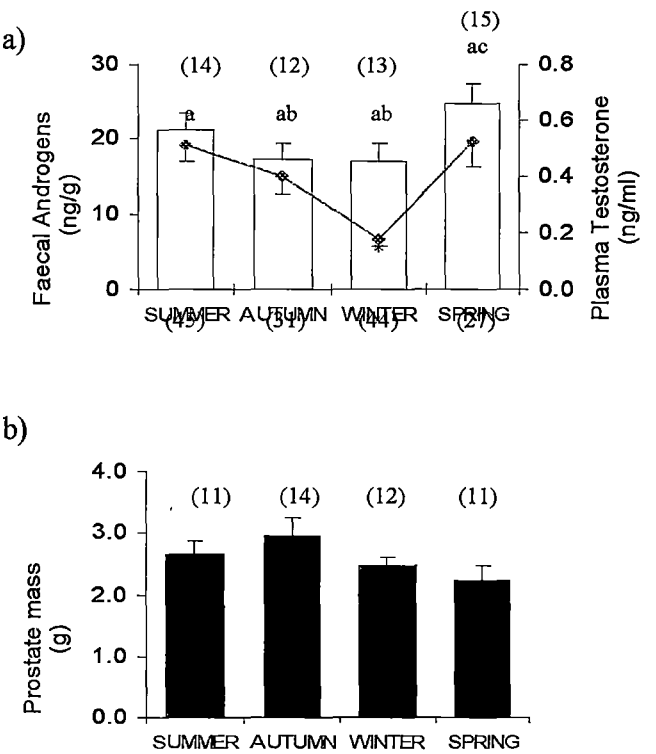


Figure 5:

Seasonal changes in average a) plasma testosterone (ng/ml) and faecal androgen concentrations (ng/g), and b) prostate mass (g) of free-ranging Tasmanian devils. Sample sizes indicated in brackets above for faecal, below for plasma. Significant differences ($P < 0.05$) indicated by an asterix (*) for plasma; values with different letters indicate significant differences between faecal groups.

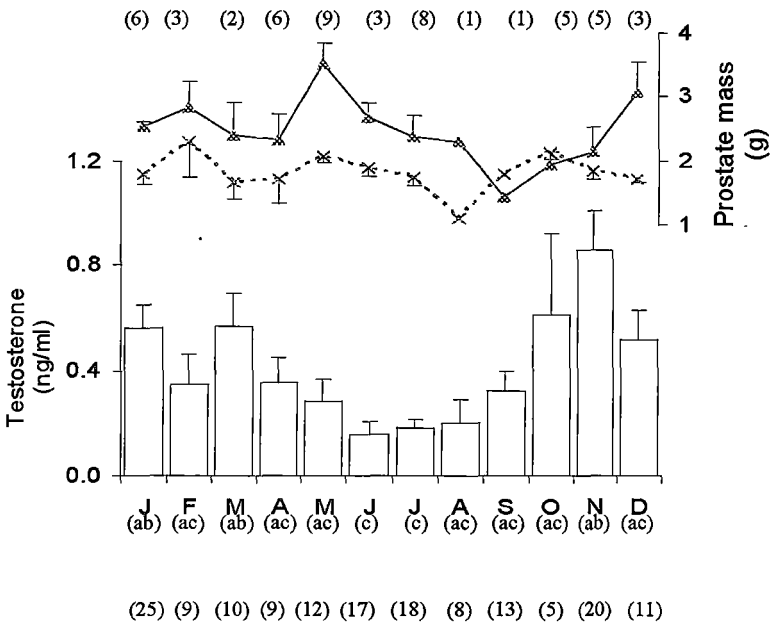


Figure 6:

Monthly changes in average plasma testosterone concentrations (ng/mL) and prostate mass (g) of adult free-ranging Tasmanian devils. Sample sizes indicated in brackets above for reproductive organs, below for plasma testosterone. Values with different letters indicate significant differences between groups for testosterone.

Table 4

Seasonal changes in mean bodymass and scrotal dimensions (width and length) of adult (2 yrs +) male Tasmanian devils trapped at Freycinet NP (FNP) and Meander district (MM) Asterix indicates significant differences within sites; values with different letters indicate differences between seasons.

	n	Bodymass (kg)	n	Scrotal width (mm)	Scrotal length (mm)
MM					
Summer	31	9.0 ± 0.23^{ab}	31	36.0 ± 0.54	27.7 ± 0.38
Autumn	19	8.5 ± 0.26^{b}	19	36.7 ± 0.36	28.1 ± 0.43
Winter	28	9.9 ± 0.24^{a}	28	36.7 ± 0.05	28.4 ± 0.28
Spring	20	9.8 ± 0.35^{a}	20	37.8 ± 0.83	28.1 ± 0.20
$(P < 0.05)$*		$F_{3,94} = 5.48$*		$F_{3,94} = 1.73$	$F_{3,94} = 0.97$
FNP					
Summer	52	10.0 ± 0.24	47	38.5 ± 0.42^{a}	29.2 ± 0.30^{a}
Autumn	61	9.8 ± 0.21	60	37.6 ± 0.38^{a}	29.4 ± 0.30^{a}
Winter	156	9.6 ± 0.15	154	36.0 ± 0.32^{b}	27.9 ± 0.91^{b}
Spring	31	10.0 ± 0.25	30	37.5 ± 0.49^{ab}	28.2 ± 0.39^{ab}
$(P < 0.05)$*		$F_{3,296} = 0.75$		$F_{3,287} = 8.35$*	$F_{3,287} = 7.80$*

Table 5

Seasonal changes in mean mass of testes and epididymides (g) of adult free-ranging Tasmanian devils (TD) and spotted-tailed quolls (STQ).

		Testes (g) (combined mass)	Epididymides (g) (combined mass)
TD	Summer	3.2 ± 0.16 (12)	2.0 ± 0.28 (8)
	Autumn	3.3 ± 0.13 (16)	1.9 ± 0.13 (14)
	Winter	3.1 ± 0.21 (9)	1.7 ± 0.14 (10)
	Spring	3.2 ± 0.12 (10)	2.0 ± 0.09 (9)
	$(P > 0.05)$	$F_{3,43} = 0.48$	$F_{3,43} = 1.05$
STQ	Summer	2.0 ± 0.28 (7)	0.9 ± 0.14 (7)
	Autumn	2.5 ± 0.24 (11)	1.0 ± 0.08 (11)
	Winter	2.4 ± 0.26 (13)	1.0 ± 0.09 (14)
	Spring	2.1 ± 0.17 (6)	1.1 ± 0.05 (5)
	$(P > 0.05)$	$F_{3,33} = 0.69$	$F_{3,33} = 0.24$

Table 6

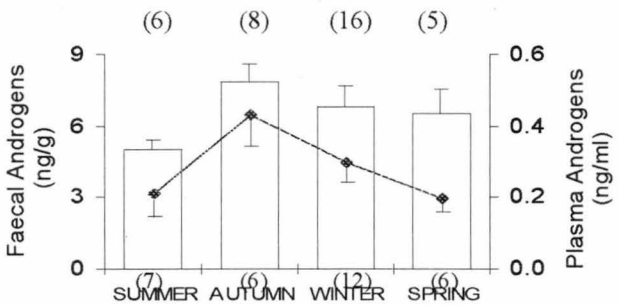
Seasonal changes in mean prostate and testes size (mm) in free-ranging Tasmanian devils (TD) and spotted-tailed quolls (STQ). Values with different letters indicate significant differences between groups.

	Testes (n)	Testes Width (mm)	Testes Length (mm)	Prostate (n)	Prostate Width (mm)	Prostate Length (mm)
TD						
Summer	12	14.1 ± 0.26	18.1 ± 0.23	11	12.4 ± 0.90	43.0 ± 2.42
Autumn	16	14.5 ± 0.21	17.8 ± 0.57	15	14.6 ± 0.63	43.0 ± 1.64
Winter	9	14.1 ± 0.88	17.2 ± 0.51	8	12.5 ± 0.18	41.4 ± 1.43
Spring	10	12.7 ± 1.00	16.9 ± 0.65	10	11.6 ± 0.58	38.9 ± 1.15
(P < 0.05)*		$F_{3,37} = 1.96$	$F_{3,37} = 0.64$		$F_{3,40} = 4.35$	$F_{3,40} = 1.14$
STQ						
Summer	7	12.2 ± 0.58	15.1 ± 0.81	5	8.4 ± 0.31 [a]	49.8 ± 3.85
Autumn	11	13.2 ± 0.43	16.0 ± 0.55	10	9.7 ± 0.50 [ab]	46.5 ± 1.23
Winter	14	12.8 ± 0.41	16.0 ± 0.56	11	10.8 ± 0.43 [b]	52.7 ± 2.28
Spring	6	12.8 ± 0 22	15.4 ± 0.22	6	8.9 ± 0.50 [a]	50.6 ± 1.83
(P < 0.05)*		$F_{3,34} = 0.82$	$F_{3,34} = 0.46$		$F_{3,28} = 4.22$*	$F_{3,34} = 2.01$

Spotted-tailed quolls

Plasma and fecal androgen concentrations averaged 0.28 ± 0.03 ng/ml, and 6.65 ± 0.51 ng/g respectively. There was a significant positive correlation between plasma and fecal androgens ($y = 9.3902x + 3.5297$, $R^2 = 0.7554$; $P < 0.001$; $n = 23$). Spotted-tailed quolls trapped in autumn, prior to their breeding season had highest concentrations of plasma testosterone ($F_{(3,27)} = 2.22$, $P > 0.05$), and matched the seasonal pattern of faecal androgens (Figure 7a) ($F_{(3,31)} = 1.05$, $P > 0.05$).

a)



b)

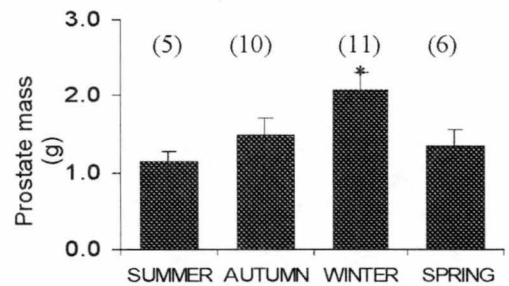


Figure 7

Seasonal changes in average a) plasma and faecal androgens concentrations (ng/g) and b) prostate mass (g) of free-ranging spotted-tailed quolls. Sample sizes indicated in brackets above for faecal, below for plasma.

Morphometric data for spotted-tailed quolls at FNP and MM were examined collectively because of the small total number of individuals captured at each site. There was no seasonal effect on bodymass (trapped: 3.1 ± 0.2 kg; $F_{(3, 22)} = 1.847$, $P > 0.05$; post-mortems: 3.5 ± 0.2 kg; $F_{(3,29)} = 0.211$, $P > 0.05$). Although there was no change in scrotal size with season in trapped males (*width* 30.7 ± 0.8 mm, $F_{(3,28)} = 0.09$, $P > 0.05$;

length 22.9 ± 0.5 mm, $F_{(3,28)} = 1.07$; $P > 0.05$), this was significant for postmortem animals (*width* 26.7 ± 0.7 mm, $F_{(3,29)} = 4.741$, $P < 0.01$; *length* 21.2 ± 0.5 mm, $F_{(3,29)} = 3.47$; $P < 0.01$), and scrotal length was greater in autumn compared to summer ($P < 0.05$) (*summer* 18.0 ± 1.3 mm, *autumn* 21.4 ± 0.7, *spring* 22.7 ± 0.8, *winter* 21.2 ± 0.5 mm). There was no effect of season on testes width or length (Table 6; $P > 0.05$), or the mass of the testes or epididymides ($P > 0.05$) (Table 5). However, the prostate was significantly wider (Table 6) and heavier (Figure 7b) in winter ($P < 0.05$). Monthly analyses were not possible due to the limited number of animals.

Spermatogenesis in quolls began from January, determined by the presence of spermatogonia and early stage spermatocytes in testicular sections. In February – March heightened activity of the epithelium became evident, with increasing cell numbers and the presence of other spermatogenic stages. From April – August, mature spermatids were released into the lumen of the seminiferous tubules and evident in the epididymis. Spermatogenic activity declined in September, with fewer representative cells; tubules remained aspermic through December.

6.4 Discussion

A key finding of this study is that the Tasmanian devil and the spotted-tailed quoll exhibit an extended reproductive season compared to other dasyurids that produce a single litter annually. Adult females and males of both species are capable of producing viable young over a longer period of the year than other species. It is likely that this broad seasonality is possible because the large body size and almost completely vertebrate diet of adult devils and spotted-tailed quolls over-ride the seasonal constraints on reproduction encountered by smaller, insectivorous species in a predictable environment.

Food dynamics are the ultimate driving force behind the timing of seasonal breeding because survival of independent young is dependent on coordinating weaning with optimal food supply (Bronson 1985). Accordingly, diet and variation in food availability is considered to have had a major influence on the evolution of marsupial life history strategies (Lee and Cockburn 1985). In carnivorous species allometric constraints of body size are reflected in dietary habits, which range from exclusive

insectivory in the smallest dasyurids through insectivory / carnivory and virtually exclusive carnivory in the largest species (Bronson 1985; Lee and Cockburn 1985). In the smaller, insectivorous dasyurids, the timing and pattern of reproduction has been shown to be associated with environmental predictability and seasonality, which strongly influences the abundance of invertebrate prey (Taggart *et al.* 1997). In contrast, mammals that feed on vertebrate prey experience less seasonal variation in food availability and nutritional content; furthermore, their energetic costs are reduced because of larger body size (Bronson 1985). While there would be some effects of drought conditions on nutritional content (e.g. fat levels), most vertebrate prey are available year round. Within the current distributional range of devils and spotted-tailed quolls, seasonal changes in their prey supply relate mainly to the annual influx of juvenile cohorts, which is synchronised with independence of marsupial carnivore young (Jones and Barmuta 1998).

Thereby, the hypothesis that environmental predictability and seasonality are major drivers of life history in carnivorous marsupials (Lee and Cockburn 1985), appears to be mainly relevant to insectivorous species. The broad seasonality of reproduction in devils and spotted-tailed quolls demonstrates that larger-bodied dasyurids with a relatively generalist diet (Jones *et al.* 2003) are not as reliant on a strong seasonal pulse in food availability, and sufficient resources are available to support lactation and rearing of late born young.

6.4.1 Timing of Breeding Events

Reproductive events in most dasyurids (Strategy I – III) typically occur during a discreet period each year, reflecting adaptation to environments with predictable and seasonal variation. In temperate regions mating and births usually occur in late autumn/winter and weaning occurs approximately 3 - 4 months later in late spring/early summer, when food resources are most abundant (Lee and Cockburn 1985; Tyndale-Biscoe 2005). The extended post-partum investment does not usually permit females to rear more than one litter a year, resulting in ecological monoestry (Lee *et al.* 1982). Time to weaning is related to body size (Russell 1982; Tyndale-Biscoe 2005); therefore, in the Tasmanian

devil onset of breeding activity occurs relatively early in the year compared to smaller dasyurids, due to an extensive duration of lactation in this species (see below).

For devils, previous studies have indicated oestrus and births are confined to a brief period in March and April, but there are a few individual cases of births occurring as late as August in the wild (Green 1967; Guiler 1970a; Hughes 1982). The present study found that breeding can commence in December - up to three months earlier than previously recorded, and that births continue through to the end of July. A sharp peak in oestrus and births was evident in February and March respectively, but the period of mating and parturition was normally distributed over a period of around six months. In captive devil populations the duration of the breeding season is similarly lengthy; females may commence oestrus as early as January (Hesterman *et al.* 2008a) and births continue through until June (Fleay 1935; Hesterman *et al.* 2008a) (see Chapter 2). For the spotted-tailed quoll, onset of oestrus and mating began in early June and continued through until August. This distribution was similar to other reports for wild (Green and Scarborough 1990; Belcher 2003) and captive quoll populations (Fleay 1940; Hesterman *et al.* 2008b) (see Chapter 3), although on the mainland reproduction may commence as early as April (Edgar 1983). At Meander, birth was recorded as late as October, which fits with observations of extended oestrous activity in captive populations (Hesterman *et al.* 2008b) (see Chapters 2, 3).

The duration of lactation lasts ~ 7 - 8 months in wild devils (Guiler 1970a), and at least 5 months in the spotted-tailed quoll (Fleay 1940; Troughton 1954; Settle 1978) (with the proviso that captive females may wean their young earlier) (Russell 1982). We found that for both species weaning commenced in December, in agreement with previous reports, but it continued until February, which was several months later than expected (Fleay 1935; 1940; Guiler 1970a; Hughes 1982, but see Kortner 2006).

6.4.2 Female Pattern of Reproduction

The wide limits of the breeding season (*i.e.* mating and births) observed here in devils and spotted-tailed quolls can be explained by two factors. Firstly, this relates to variance in the onset of breeding within populations (see also, Proximate Cues); and secondly, to characteristics of those species' oestrous cycle.

Most dasyurids do not usually reach puberty until 12 months of age, because of limitations on the suitable breeding period, and the extended duration of maternal dependence (Lee *et al.* 1982). In the present study we confirmed that female spotted-tailed quolls usually attain sexual maturity and breed at this age, whereas female devils did not usually reach puberty or produce young until the age of two years. This is in agreement with birth data from wild and captive populations of devils and spotted-tailed quolls (Fleay 1935; 1940; Guiler 1970b; Hughes 1982; Hesterman *et al.* 2008a), and longitudinal endocrine studies in captivity (Hesterman *et al.* 2008a; 2008b) (see Chapters 2, 3). The proportion of wild female devils that became sexually mature or produced young in their first year was low (5 – 10 %), as previously reported (Hughes 1982; Hawkins *et al.* 2006). Similarly, in captivity the proportion of devils that ovulate prior to the age of two is very low (2 %; n = 46; HH, unpublished data). Many marsupials breed before they attain maximum mass (Cockburn 1997), but Lee *et al.* (1982) proposed that a delay in onset of sexual maturity in the devil could be related to the species' considerably larger body size than other dasyurids. In support of this hypothesis the present study found that precocious breeding was significantly related to body mass, and occurred significantly later in the year. Of interest, Belcher's (2003) study of spotted-tailed quolls noted that females did not usually produce young until two years of age, but he did not indicate whether they attained puberty. Without this information it is difficult to determine if the delay to breed was the result of sexual immaturity or failed mating.

An evident second factor in the wide distribution of births in devils and spotted-tailed quolls is facultative polyoestry. Facultative polyoestry is characteristic of Strategy III dasyurids, and viewed as an adaptive strategy to maximise lifetime reproductive success by guarding against reproductive failure or limited resources (Lee *et al.* 1982; Tyndale-Biscoe 1984). This feature has previously been inferred for spotted-tailed quolls (Edgar 1983; Collins *et al.* 1993), but remained an uncertain strategy for devils (Lee *et al.* 1982; Krajewski *et al.* 2000; McAllan 2003). Recent endocrine studies of captive populations confirmed that both species are facultatively polyoestrous and can undergo two oestrous cycles during the breeding season (Hesterman *et al.* 2008a; 2008b) (see Chapters 2, 3). In the present study several female devils that were re-trapped were experiencing a

second oestrus and others had replaced a lost litter. This data supports the notion that natural occurrence of polyestry is probably low (Tyndale-Biscoe 1984; Bryant 1986), because most females bred successfully at the beginning of the season.

Because devils and quolls have a typically short reproductive life-span as for other dasyurids (Cockburn 1997), it is advantageous both to fit in an extra litter by breeding at a younger age if possible, and to replace lost litters. If young born later in the season had a much reduced chance of survival, we would expect selection against the less strictly regulated season and polyoestrous trait in devils and spotted-tailed quolls.

Weaning plays an important role in timing of breeding because, like other dasyurids (Tyndale-Biscoe and Renfree 1987), devils and spotted-tailed quolls experience lactational anoestrous (Hesterman *et al.* 2008a; 2008b) (see Chapters 2, 3). Despite the wide distribution in timing of births for devils and quolls in our study populations, nearly all females had completed lactation by the end of summer. Predictably, this is the period of prey abundance for both species, when predator-naïve juveniles of most prey species become independent (Belcher 1995; Jones and Barmuta 1998). Findings here suggest that later breeding devils wean their young earlier to take advantage of these optimal conditions. Lactation length is known to vary in other dasyurids (Merchant *et al.* 1984; Soderquist and Serena 1990; Cockburn 1992), and this strategy would ensure a level of synchrony within devil populations is maintained. For quolls, there is no need to wean young early because females have a period of at least five months prior to the onset of the next breeding season.

6.4.3 Male Pattern of Reproduction

Length and timing of male reproductive effort is integral to understanding dasyurid life history strategies (Lee *et al.* 1982), as their breeding patterns are strongly influenced by those of the female (Sadlier 1969; Tyndale-Biscoe 2005). The reasons that male devils and spotted-tailed quolls maintained reproductive readiness for such a prolonged duration are clearly related to the extensive parameters of annual oestrous activity found in these populations. Although the duration of reproductive readiness for males was comparatively extensive, males did not remain fertile year-round, in common with most marsupials (Tyndale-Biscoe and Renfree 1987).

As for other male mammals, in dasyurids a seasonal elevation in androgen concentrations is associated with breeding activity. In free-ranging devils and spotted-tailed quolls androgens increased several months prior to the main breeding season to encompass the first onset of oestrous activity in individual females; and concentrations were sustained for several months well into the mating period. This pattern is also observed in captive populations (Hesterman and Jones 2008) (see Chapters 2, 3), and in contrast to other iteroparous dasyurids, where there is a brief rise only and the peak occurs during the main breeding season (Tyndale-Biscoe and Renfree 1987). Elevated androgens are not essential for the maintenance of spermatogenesis (Inns 1982; Bryant 1986; Woolley 1990b) and can also result from intra-specific aggression and sexual encounters during the breeding season (Inns 1982; Bryant 1986; Gemmell *et al.* 1986), which may account for this observation in devils and spotted-tailed quolls which exhibit an extended period of mating activity.

The effect of androgens on development of the prostate was evident for devils and spotted-tailed quolls, and the organ typically peaked in size and mass by the onset of the main mating period (Tyndale-Biscoe and Renfree 1987). The seasonal changes of the prostate, testes or epididymides in the devil or the spotted-tailed quoll were, however, conservative compared to the transformation observed in most other monoestrous dasyurids (Tyndale-Biscoe and Renfree 1987). Spermiogenesis was confirmed to last for nine months from November to August in adult male devils, expanding on preliminary observations by Sharman (1959) and Hughes (1982). Spotted-tailed quolls produced sperm from April to August/September, similar to that observed in the eastern quoll (Fletcher 1985a). The duration of sperm production was similar to the period of spermatorrhoea in other polyoestrous dasyurids, including species that produce two litters a year (McAllan 2003). This strategy would be necessary to ensure opportunities for fertilisation are maximised, and fits with observations for an extended oestrous period in the devil and spotted-tailed quoll.

Costs are associated with priming of the reproductive tract, production/maintenance of spermatogenesis, and energy contributions toward behavioural activities for procurement and retention of mates (*e.g.* territory establishment, copulation and mate-guarding). Accordingly, male strategies have evolved to offset such costs, including restricting the

period of fertility and varying the length of sperm storage (Tyndale-Biscoe and Renfree 1987; Taggart and Temple-Smith 1994; Tyndale-Biscoe 2005). Smaller species may incur substantial energetic costs associated with the production of sperm and seminal plasma from the prostate (Taggart *et al.* 2003; Tyndale-Biscoe 2005), and male effort is critically intense in semelparous species (Lee *et al.* 1982; Lee and Cockburn 1985). The ability for species such as devils and spotted-tailed quolls to maintain spermatogenesis over a protracted period supports the assertion that breeding incurs considerably lower reproductive effort in iteroparous dasyurids (Lee *et al.* 1982; Lee and Cockburn 1985; Taggart and Temple-Smith 1994; Taggart *et al.* 1997).

6.4.4 The Role of Proximate Cues

In temperate regions, photoperiod is the most widely used proximate cue for timing onset of behavioural and physiological processes associated with copulation and birth (Sadlier 1969; Bronson 1985). Response to annual change in day length is well documented for a range of eutherian mammals (Sadlier 1969), and found to be the primary cue for several marsupial species that have been experimentally tested (Tyndale-Biscoe and Renfree 1987; Hinds and Loudon 1997; McAllan 2003). The influence of photoperiod on the timing of breeding in devils and spotted-tailed quolls is unknown, but presumably these species respond to different components of changing day length as onset of breeding occurs at different times of year. Of interest was our finding that some female devils enter oestrus in December/January and produce young up to two months prior to the mean birth date for the population. Evidently, males have also achieved fertility by this time. The primary stimulus for onset of breeding likely acts at a population level on species (Sadlier 1969, but see Bronson 1985) to bring all females into a pre-oestrous state; however, other factors such as internal regulators of the ovarian cycle (*e.g.* physiological maturation, lactational suppression of follicular activity), determine which individuals will achieve an oestrus state, and when this will occur (Sadlier 1969).

Additional proximate cues implicated in activation and synchronisation of oestrous cycles in other dasyurid species include localised factors from altitude and rainfall, to inter-specific competition and social stimulus (McAllan 2003; Tyndale-Biscoe 2005).

Soderquist (1993a) proposed that the level of synchrony will be enhanced in species that occur in higher densities and are more sociable, which fits the observations of the present study. For devils, despite some flexibility in the onset of breeding there was remarkably little variation in mean birth week either between study sites or in different years, similar to Guiler's findings (1970b). By contrast, data for spotted-tailed quolls showed no apparent peak in births, which were instead staggered over a three month period, as Belcher (2003) observed.

The devil and spotted-tailed quoll are both solitary species (Russell 1984) that occupy large home ranges (Pemberton 1990; Belcher and Darrant 2004; Körtner *et al.* 2004; Claridge *et al.* 2005; Glen and Dickman 2006 MJ, unpublished data), but they contrast in their degree of sociality and in their spatial organisation. In spotted-tailed quolls, male ranges overlap those of other adult males and females but females maintain exclusive territories (Belcher and Darrant 2004; Körtner *et al.* 2004; Claridge *et al.* 2005; Glen and Dickman 2006). By contrast, devils of both sexes have broadly overlapping home ranges (Pemberton 1990 MJ, unpublished data) and are more social, forming temporary feeding associations (Buchmann and Guiler 1977; Pemberton and Renouf 1993). Devils are ecologically dominant and so are much more abundant than spotted-tailed quolls (Jones and Barmuta 1998). Given the lower density and more extensive spacing of spotted-tailed quolls in the landscape, availability of males would be relatively limited, therefore it would be disadvantageous for females to exhibit a highly synchronised oestrus.

6.4.5 Conclusions

The key evolutionary force driving timing of reproduction in seasonally breeding animals is the need to produce offspring within an optimal time of year to survive; and this is primarily driven by food availability (Bronson 1985; Tyndale-Biscoe 2005). Body size (which places limitations on the prey size of carnivores along an insectivory/carnivory continuum) and diet, are major determinants that influence longevity; and all of these factors strongly influence the evolution of mammalian reproductive strategies (Bronson 1985; Cockburn and Johnston 1985; Promislow and Harvey 1990). Thus, it should not be unexpected that variance can arise even within Families that inhabit a similar environment, as they may be differently dependent or

affected by ambient and local cues (Bronson 1985). This study has demonstrated that, while the ultimate factor – synchrony of independence with maximum food availability – is undoubtedly important in timing of reproductive events, it may be relaxed in species where ecological and reproductive attributes permit a level of flexibility. In devils and spotted-tailed quolls these are large body size, generalist flesh-eating diet, facultative polyoestry and variation in the length of lactation.

For carnivores that feed on vertebrate prey there is less seasonal and nutritional variance in food; therefore, costs associated with reproduction are likely to be primarily energetic (Bronson 1985). Larger-bodied mammals also have reduced overall energetic costs associated with reproduction because they have lower metabolic rates, thermoregulatory costs and greater fat stores than smaller species (Bronson 1985). Collectively, consideration of body size and diet can serve as useful predictors of annual breeding patterns, and this can be augmented by more intimate knowledge of species characteristics such as social and spatial organisation.

Tasmanian devils and spotted-tailed quolls do share many features of their reproductive life history with other seasonally breeding, iteroparous members of the family Dasyuridae. Because the devil can breed at 12 months of age and is facultatively polyoestrous, this species conforms to all of the characters used to assign Strategy III dasyurids; and as such should be positioned therein, alongside the spotted-tailed quoll. This study highlights the need to view dasyurid life history strategies as more of an adaptive continuum which has led to the remarkable level of diversity and success of this marsupial group.

6.4.6 Applications to Conservation

Changes in the anatomy/histology of the female reproductive tract during the breeding season paralleled those previously detailed for the devil (Hughes 1982) and other dasyurids (Tyndale-Biscoe and Renfree 1987; Woolley 1990a). Ovarian dynamics and breeding status of females were reflected in plasma progesterone concentrations and vaginal cytology, which have been successfully applied to monitor reproduction in a range of marsupials (Tyndale-Biscoe and Renfree 1987). Measurement of urinary and faecal hormones have also been measured to evaluate reproductive status of wildlife, and

provide an attractive, non-invasive alternative for monitoring both captive and free-ranging populations (Lasley and Kirkpatrick 1991; Schwarzenberger *et al.* 1996; Monfort 2003). This is becoming an increasingly popular technique for a range of marsupials, which now include the devil and spotted-tailed quoll (Hamilton *et al.* 2000; Stead-Richardson *et al.* 2001; Bradshaw *et al.* 2004; Woodd *et al.* 2006; Hesterman *et al.* 2008a; 2008b) (see Chapters 2, 3). However, there is only one other published account of its application in free-ranging populations, and that was to study seasonality in male wombats (Hamilton *et al.* 2000). In the present study, faecal sex steroids were successfully measured in male and female devils and spotted-tailed quolls, and shown to be correlated with plasma hormones. However, results indicate that the application of endocrinology alone to evaluate ovarian dynamics in free-ranging dasyurid populations is constrained by the typically brief duration of oestrus and gestation. Changes in pouch appearance have been noted as a general indicator of reproductive condition in dasyurids (Fleay 1935; 1940; Woolley 1974; Tyndale-Biscoe and Renfree 1987), and associated with changes in plasma and faecal sex steroid concentrations in captive devils and spotted-tailed quolls (Chapter 4) (Hesterman *et al.* 2008c). Here, this information was further validated by linking pouch condition with development of the vaginal complex, and building on early observations by O'Donoghue (1911). This research confirms pouch assessment as a ready and practical, non-invasive technique that can assist in monitoring reproductive status and seasonality in wild devil and spotted-tailed quoll populations by confirming events such as puberty, oestrus and ovulation; and may be extended to other dasyurid species.

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CHAPTER 7
GENERAL DISCUSSION

The Tasmanian devil and the spotted-tailed quoll are the largest extant carnivorous marsupials, following the relatively recent extinction of the Thylacine (*Thylacinus cyanocephalus*) circa mid 1930s (Jones *et al.* 2003). Devils and spotted-tailed quolls have always attracted scientific interest, perhaps because of their conspicuous presence and a history of human interactions, resulting from their habits of predation on livestock. Earlier and more recent studies on these two species have focused on exploring and understanding ecological attributes such as diet, population dynamics and behaviours (Fleay 1952; Green 1967; Guiler 1970b; Eisenburg *et al.* 1975; Buchmann and Guiler 1977; Mansergh 1984; Green and Scarborough 1990; Pemberton 1990; Pemberton and Renouf 1993; Belcher 1995; Jones and Barmuta 1998; 2000; Belcher 2003; Belcher and Darrant 2004; Körtner *et al.* 2004; Claridge *et al.* 2005). Yet, while we are well aware that the two fields of ecology and reproductive biology are inextricably linked (Rowlands and Weir 1984; Tyndale-Biscoe 1984; Bronson 1985; Tyndale-Biscoe and Renfree 1987), the latter discipline appears to have been largely overlooked for the devil and spotted-tailed quoll.

Today, a rapidly changing environment has prompted a shift toward investigating wildlife distribution and dynamics to examine the effects of anthropogenic perturbations, to inform and guide the preservation of populations. A multidisciplinary, broader approach to reproductive sciences is required to meet this more unified goal, and gathering such information should be considered as a priority rather than a late endeavour (Temple-Smith 2003; Wildt *et al.* 2003).

This PhD research has extended our knowledge of marsupial reproductive physiology, and enhanced appreciation of the diversity of patterns that have evolved, particularly within the Dasyuridae. The study has provided necessary (and timely) information that has practical applications for improved monitoring and management of captive and free-ranging populations of the largest remaining marsupial carnivores. Following is a review of my key findings on the intricacies of reproductive processes in the Tasmanian

devil and spotted-tailed quoll as these pertain to a broader understanding of dasyurid and marsupial strategies; and the implications of this study for conservation management.

7.1 Reproductive Physiology of the Devil and Spotted-tailed Quoll

This study successfully characterised the reproductive cycle of the female Tasmanian devil and spotted-tailed quoll by measuring plasma and faecal sex steroids, as well as other methods such as gross and histological changes of the reproductive organs, and assessment of pouch appearance. A complementary approach was taken to also gain a better understanding of the breeding processes and seasonality in the male devil and spotted-tailed quoll. This was achieved through a combination of sex steroid monitoring, and assessment of anatomical and/or histological changes in the testes, epididymides and prostate to determine aspects of fertility. Measurement of faecal steroids for these species was validated through laboratory and physiological methods to demonstrate correlation between plasma hormones and their faecal metabolites; and, for females, correlation with vaginal cytology and association with reproductive events such as copulation and birth.

7.1.1 The Female

The oestrous cycles of the devil and spotted-tailed quoll were similar in many respects to that observed in other dasyurids, including the pattern of sex steroids, changes in vaginal cytology, pouch appearance and development of the reproductive tract. Monitoring of hormone concentrations allowed specific characteristics including annual pattern of oestrous, the mechanism and timing of ovulation and gestation length to be effectively detailed. Endocrine studies were further validated through assessment of urogenital sinus smears and observations of mating activity, and for devils, confirmation of births.

The pattern of faecal oestrogens in the devil and spotted-tailed quoll reflected enhanced follicular activity at oestrus, similar to observations of plasma oestradiol/faecal oestrogens in other marsupials (Tyndale-Biscoe and Renfree 1987; Bradshaw and Bradshaw 1992; Millis *et al.* 1999; Johnston *et al.* 2000). There was a biphasic pattern of plasma progesterone/faecal progestagens in both species consisting of a brief, minor rise associated with oestrus followed by a major, sustained rise during the luteal phase.

This pro-oestrous progesterone surge is also observed in the eastern quoll and kowari, and proposed to originate from developing or luteinised ovarian follicles (Fletcher 1985b, Hinds and Selwood 1990). Longitudinal endocrine monitoring of other dasyurids is required to confirm if this progesterone pattern is a family-shared trait. Changes in progesterone/progestagen have only been monitored in two other dasyurid species and sampling was insufficient to detect a pro-oestrous rise (*Antechinus stuartii*: Hinds and Selwood 1990; *Phascogale tapoatafa*: Millis *et al.* 1999). In other marsupials, progesterone/progestagen concentrations do not increase until after ovulation when corpora lutea have formed (Tyndale-Biscoe and Renfree 1987; Johnston *et al.* 2000; Millis and Bradley 2001; Paris *et al.* 2002; West *et al.* 2004; Finlayson *et al.* 2006; Woodd *et al.* 2006).

This study demonstrated that the devil and spotted-tailed quoll conform to the basic breeding pattern of other non-macropod marsupials, whereby lactation suppresses further ovarian activity (Tyndale-Biscoe and Renfree 1987). Both species ovulate spontaneously, but it was of particular interest that this is not always the case for quolls: this species exhibited significant differences in the duration of the non-mated and mated oestrous cycle. There are numerous eutherian species which are induced, or "reflex" ovulators (Rowlands and Weir 1984), but the koala (*Phascolarctos cinereus*) is the only marsupial known to exhibit this trait (Johnston *et al.* 2000). It is possible that proximate cues other than the physical act of coitus may be involved in inducing a luteal phase in the spotted-tailed quoll; considering studies of the brush-tailed bettong (*Bettongia penicillata*) (Hinds and Smith 1992) and American opossum (*Monodelphis domestica*) (Hinds *et al.* 1992) indicate that the mere presence of a male can induce oestrus. The adaptive benefits of a degree of reflex ovulation in some marsupials could relate to improved chances of fertilisation where male availability is limited by female predilections or population density (see Reproductive Strategies; Section 7.2).

While photoperiod is likely to be the main environmental cue influencing the onset of breeding, other such exogenous cues are evidently involved in fine-tuning of mammalian oestrous cycles (Sadlier 1969). There is evidence that social cues such as chemical communication (e.g. pheromones) are involved in timing of oestrous cycles in various mammalian species (Sadlier 1969), including dasyurids (Croft 1982). For

example, Scott (1986) found that in *A. stuartii*, isolation of females results in loss of their typically high degree of reproductive synchrony unless they are exposed to olfactory cues from other females. It is possible that in the present study, isolation of solitary female devils and quolls may have prevented some individuals from entering oestrus by limiting some necessary chemical stimulation.

There was a lengthy interval of approximately a week between oestrus and ovulation in the devil and spotted-tailed quoll, which is a feature that has been documented for several other dasyurids (Hill and O'Donoghue 1913; Selwood 1980; Fletcher 1985b; Hinds 1989), but is not apparent in other marsupials (Tyndale-Biscoe and Renfree 1987). Copulations also occurred over a variable interval, indicating that ovulation does not occur at a fixed time relative to oestrus. Extended periods of sperm storage are known to occur in other dasyurids (reviewed in Taggart *et al.* 2003), and, collectively, these results suggest that inter-male sperm competition is prevalent in this Family.

The pattern of plasma progesterone/faecal progestagens followed that of other dasyurids and marsupials, with sustained concentrations reflecting the development of the corpora lutea, and a rapid decline in concentrations associated with parturition or termination of the non-pregnant luteal phase. Typical of other marsupials, there was no qualitative or quantitative difference in plasma progesterone profiles between the mated and non-mated oestrous cycle in the devil and spotted-tailed quoll (Tyndale-Biscoe and Renfree 1987; Hinds 1989; 1990; Hinds and Selwood 1990). For devils, faecal steroid profiles did differ between mated and non-mated females during periods (in pregnant animals) associated with blastocyst expansion and implantation (Hinds and Selwood 1990), suggesting maternal recognition of pregnancy may occur in this species. In marsupials the placenta is considered to have an insignificant role in endocrine regulation during pregnancy due to the relative autonomy of the corpora luteum (Tyndale-Biscoe and Renfree 1987); but, Hinds (1990) notes that the yolk sac placenta is capable of converting precursors such as pregnenolone, and metabolising other steroids in the tammar wallaby. The endocrine role of the feto-placental unit of non-macropods, if any, is unknown, but this could offer an explanation for the differences observed in the pattern of excretion between plasma progesterone and its faecal metabolites in the devil.

For dasyurids, gestation (≈ luteal phase) has often been inferred using the time lapsed between copulation and birth. However, because matings may occur over a protracted period of up to a week, and there is the variable interval to ovulation and period of sperm storage, this method is rather unsuitable for dasyurids (Tyndale-Biscoe and Renfree 1987). Endocrine profiling of devils and spotted-tailed quolls allowed systematic monitoring of ovarian dynamics and accurate measurement of the follicular and luteal phase. This meant the gestation period was accurately determined for the devil; for the spotted-tailed quoll, inability to confirm birth in any of the mated females precluded documenting the information for that species. Longitudinal monitoring of endocrine function is important to determine these parameters of the ovarian cycle and the existence of traits such as a variable ovulatory interval; and warrants extension to more members of this Family. The characteristically brief oestrous of marsupials (Tyndale-Biscoe and Renfree 1987) necessitates frequent sampling to monitor individual cycles, so faecal endocrinology may be a preferred option to plasma collection for assay.

Like most dasyurids (with the notable exception of *Antechinus*) (Tyndale-Biscoe and Renfree 1987; Hinds 1989; Hinds and Selwood 1990) devils and spotted-tailed quolls were confirmed to be seasonally, facultatively polyoestrous. Endocrine profiles revealed that females of both species experience a delay between successive oestrous cycles. This feature has also been observed for the kowari (Fletcher 1985b). In the eastern quoll, however, there is no such lapse; and unmated females routinely fit an additional, third oestrous cycle into the breeding season (Hinds 1989). No delay is noted to occur between cycles in members of the Family Dasyuridae that produce several litters annually (e.g. *Sminthopsis*, Woolley 1990a).

A key finding of the present study was that pouch appearance in the devil and spotted-tailed quoll proved to be a reliable indicator of reproductive condition. Marked changes in pouch appearance (reddening, enlargement) during the breeding season are typical of dasyurids, including the devil and spotted-tailed quoll (Fleay 1935; 1940; Woolley 1974; Fletcher 1985b; Soderquist and Serena 1990; McAllan *et al.* 1991; Woolley 1991; Oakwood 2000; Selwood and Cui 2006), but less apparent in other marsupial species (Tyndale-Biscoe and Renfree 1987). Early evidence showed that in the eastern quoll, development of the pouch and corpora lutea are linked (O'Donoghue 1911), but there

have been no further attempts in any dasyurids to investigate physiological correlates of pouch development during the breeding season. Here, concurrent evaluation of endocrinology and vaginal cytology demonstrated that the readily identifiable changes in pouch appearance are associated with specific stages of the oestrous cycle. These findings were unequivocally confirmed through post-mortem gross examination of reproductive tracts. Pouch development in devils and spotted-tailed quolls was associated with important stages in the reproductive cycle such as onset of puberty, oestrus and post-ovulation. This has important implications as an alternative method for monitoring of *in situ* and *ex situ* populations (see Implications for Captive Breeding and Conservation; Section 7.3).

7.1.2 The Male

The male can be considered an 'ecological factor' of reproduction (Sadlier 1969), in terms of their effect on the female in the stimulation of oestrus in some mammalian species, as well as an obvious role in fertility. Documenting male testicular-endocrine rhythms is a necessary step towards an improved understanding of breeding synchrony between the sexes. Linking features of the male's biology including changes in sex steroids (androgens), testes and accessory glands and the period of spermatogenesis allows an enhanced understanding for more accurate interpretation of species' reproductive strategies and patterns.

The period of fertility in the male is often inferred relative to the timing of breeding in the female (Sadlier 1969). Understandably, because the mating season is relatively brief in most members of the Dasyuridae (Lee and Cockburn 1985), monitoring has tended to specifically encompass the period of the year associated with mating activity. The pattern of androgen concentrations during the breeding season have previously been measured for several dasyurids (Kerr and Hedger 1983; Bradley 1987; Tyndale-Biscoe and Renfree 1987; Schmitt *et al.* 1989; Millis *et al.* 1999), including other iteroparous species such as the eastern quoll and *Sminthopsis* (McDonald *et al.* 1981; Bryant 1986; Woolley 1990b). In this study, longitudinal monitoring of devils and spotted-tailed quolls was undertaken throughout the year, which proved to be invaluable for correlating male hormone profiles with the period of ovarian activity in females.

Although both the Tasmanian devil and the spotted-tailed quoll were confirmed to be seasonal breeders, as are the majority of dasyurids (McAllan 2003), the duration of the annual mating period in both captive and free-ranging populations of both species is months longer than previously recognised (Fleay 1940; Green 1967; Guiler 1970a; Hughes 1982; Belcher 2003). The onset of oestrous activity and births in devils began in December - up to three months in advance of the expected timeframe - and continued through until mid-June. For spotted-tailed quolls, oestrous commenced in the anticipated month of May but continued as late as October with a record of birth occurring during that month.

For male devils and spotted-tailed quolls, the annual reproductive cycle encompassed that of the female, which is the common pattern for seasonal breeders (Bronson 1989; Tyndale-Biscoe 2005). Seasonal initiation of breeding activity commences with physiological processes that prime the male, because production of mature sperm can take up to several months (Tyndale-Biscoe and Renfree 1987). The onset of activity was evidenced through a rise in plasma and faecal androgen concentrations; this was accompanied by commencement of spermatogenesis and development of the prostate in advance of the main mating period. This is typical of the sequence of changes observed in seasonally breeding male mammals (Rowlands and Weir 1984), including dasyurids (Tyndale-Biscoe and Renfree 1987). The period of spermatogenesis neatly encompassed the outer limits of oestrous activity and births in devil and spotted-tailed quoll populations.

The major contrast between male spotted-tailed quolls and devils and other members of the Family Dasyuridae was the pattern of plasma and faecal androgen concentrations. Androgen concentrations peaked prior to, rather than during, the main mating period, and remained elevated for a prolonged time over several months. By contrast, even in *Sminthopsis*, which have an extended mating season of up to nine months, plasma androgen concentrations are not sustained (McDonald *et al.* 1981; Woolley 1990b). In devils and spotted-tailed quolls, the sustained elevated androgen concentrations are likely to ensure males remain behaviourally and physiologically capable of procuring/fertilising females undergoing oestrous cycles late in the season.

7.2 Reproductive Strategies of the Dasyuridae

The evolution of life history characteristics in the Dasyuridae represent a combined response to the physical, energetic and social environment; this has had a profound effect on the development of distinct breeding strategies in this taxon (Lee *et al.* 1982; Krajewski *et al.* 2000). In this study, detailed information was collected on the reproductive physiology, breeding patterns and demographics of devil and spotted-tailed quoll populations. Previous research has concentrated on insectivorous species and has indicated that environmental predictability and seasonality are the main factors involved in moulding reproductive strategies in dasyurids (Lee and Cockburn 1985; Taggart *et al.* 1997). My results show that diet and body size also markedly influence annual breeding patterns in the Dasyuridae, and verify that other species-specific traits, such as spatial organisation, can also be important.

A major finding of this study was that in Tasmania, the devil and the spotted-tailed quoll have an extended reproductive season compared to all other dasyurids that produce a single litter each year. Reproductive activity (oestrus, births and lactation) in spotted-tailed quoll and devil populations took place for up to nine and twelve months, respectively. The length of this period is more comparable with the seasonal duration of breeding in obligate polyoestrous dasyurids such as Sminthopsis (Strategy IV). For devils and spotted-tailed quolls, reproductive flexibility was apparently related to characteristic features of their reproductive biology, such as individual variation to onset of oestrus, facultative polyoestry, a variable inter-oestrous interval (see Hesterman *et al.* 2008a; 2008b) (Chapters 2, 3) and variation in the length of lactation; as well as ecological attributes of these species, including social and spatial organisation.

Facultative polyoestry is likely to have played some role in the spread of births observed here for the devil and spotted-tailed quoll, but proximate factors are considered to have also had a marked effect on the timing of breeding. Timing of reproduction in mammals is influenced by a host of proximate factors from dietary and social cues to environmental features such as photoperiod and rainfall (Sadlier 1969; Bronson 1985; Tyndale-Biscoe and Renfree 1987; Bronson 1989; McAllan 2003). Photoperiod is a likely candidate influencing seasonality because these species inhabit a temperate region

(Sadlier 1969; Bronson 1985; Hinds and Loudon 1997); however, a second feature was the influence of additional cues, acting both at the level of the species and the individual.

For devils, the annual timing of mating was predictable across years and relatively co-ordinated within and between populations, compared to spotted-tailed quolls. Findings of this study, taken collectively with comparative findings on these species' ecology and behaviour (Buchmann and Guiler 1977; Pemberton and Renouf 1993; Jones and Barmuta 1998; Belcher and Darrant 2004; Körtner *et al.* 2004; Claridge *et al.* 2005; Glen and Dickman 2006), suggest that proximate cues are involved in synchronisation (and stimulation) of oestrous cycles for both species. The hypothesis that timing of oestrus is related to population density and spacing – and hence, availability of males (Soderquist 1993b), was also borne out, with spotted-tailed quolls, which occur in lower density to devils and have female territoriality (devils are not territorial), showing less synchrony in births. Some variation in the timing of oestrus between individuals of each species was evident, and may allow females to improve their fitness by increasing the chance of engaging 'preferred' males in the population (Belcher and Darrant 2004).

Data from free-ranging devil and spotted-tailed quoll populations showed that timing of weaning was much more synchronous than parturition, and occurred over a relatively brief period (Dec – Feb), around the same time of year to other dasyurids inhabiting a similar environment (Lee and Cockburn 1985; Tyndale-Biscoe 2005). The optimal period for reproduction is obviously seasonally regulated in these species, and likely driven by prey abundance (Belcher 1995; Jones and Barmuta 1998). Dasyurids are known to individually vary their lactation length (Merchant *et al.* 1984; Soderquist and Serena 1990; Cockburn 1992). It is likely that this trait allowed devils and quolls to wean their late-born litters prematurely. This finding suggests that it is strongly advantageous to wean young within that particular window of the year even though this is not obligate.

The physical environment is the main factor that determines whether reproduction will be seasonal or continuous, but in either case food availability is the ultimate factor that delineates these parameters because all animals must produce offspring at a time of year that optimises survival (Bronson 1985; Tyndale-Biscoe 2005). Lee and Cockburn (1985)

proposed that environmental predictability and seasonality are the key drivers of life history in carnivorous marsupials, but this prediction was made based on studies of insectivorous species; in which reproductive patterns are strongly influenced by the abundance of invertebrate prey (Taggart *et al.* 1997). As well as the degree of environmental seasonality, body size and diet exert a major influence on species' breeding patterns (Bronson 1985; Lee and Cockburn 1985; Bronson 1989; Taggart *et al.* 1997). Findings here suggest that large body size and a predominantly vertebrate diet buffer the devil and spotted-tailed quoll from the seasonal constraints on reproduction experienced by smaller, insectivorous dasyurids in a predictable, temperate environment. The energetic requirements of late lactation are substantial for marsupials (Tyndale-Biscoe 2005). There is much less seasonal variation in availability and nutritional content of vertebrate than invertebrate prey. A lack of evidence for extended/late breeding in other facultatively polyoestrous species in a similar habitat, such as eastern quolls, provides support for this hypothesis. At 0.7 – 1.3 kg in body mass (Strahan 2005), eastern quolls are sufficiently small that they are primarily insectivorous in most habitats, although their diet includes a greater proportion of vertebrate prey in alpine areas (Blackhall 1980; Jones and Barmuta 1998).

The breeding strategies exhibited by dasyurids operate within phylogenetic constraints, which include a degree of opportunism for most species, such as the occurrence of facultative polyoestry (Lee *et al.* 1982; Lee and Cockburn 1985). This is not surprising, given the typically short reproductive life span of dasyurids (Cockburn 1997). For devils and spotted-tailed quolls, the present study also demonstrated marked variation in timing of breeding events and determined that while precocial breeding (at 12 months of age) is not common in female devils, it can allow individuals that attain a good body mass to produce an additional litter in their life. This is an example of how bioenergetic influence reaches beyond merely establishing the limits of the breeding season, but is also involved in modifications of breeding patterns (Bronson 1985). The male also incurs costs associated with maintaining reproductive capability (Tyndale-Biscoe 2005); and an indication of this was that, despite the lengthy duration of mating activity in spotted-tailed quoll - and particularly, devil populations - males did not maintain elevated androgen concentrations or fertility throughout the year.

In summary, it was determined that while Tasmanian devils and spotted-tailed quolls express many of the reproductive traits common to dasyurids, both species did exhibit some unique differences in features of their reproductive endocrinology and breeding patterns. Findings have shown that devils belong in the same category as the spotted-tailed quoll (Strategy III), and illustrate species-specific complexities which propose marsupial life history strategies actually operate along more of a continuum that previously determined.

7.3 Implications for Captive Breeding and Conservation

It is widely accepted that on a global scale, fauna is in the grip of an extinction crisis, largely because of human-related activities. As a response, conservation programs have been established to try to compensate and preserve biodiversity in the wild – primarily these activities centre around arguably more charismatic vertebrates, and are focused on mammalian species (Olney *et al.* 1994). Effective conservation necessitates an integrated and multi-disciplinary approach to the preservation of wild and captive populations (Olney *et al.* 1994; Wildt *et al.* 2003). There are a number of internationally recognised 'flagship' mammalian species which stand as testimony to such efforts, including the Californian condor (*Gymnogyps californianus*) (Toone and Wallace 1994), golden lion tamarins (*Leontopithecus*) (Dietz *et al.* 1994), and the black-footed ferret (*Mustela nigripes*) (Toone and Wallace 1994; Howard *et al.* 2003).

Compared to other mammals, Australian marsupials have undergone an unprecedented wave of recent extinctions, and similarly are subject to continued decline because of a multitude of anthropogenic causes from habitat modification through to introduction of foreign species (Fletcher and Morris 2003; Jones *et al.* 2003; Temple-Smith 2003). Conservation actions and recovery programs have been initiated for a range of different marsupials, but further development of comprehensive information on species' reproductive biology is required to improve outcomes (Temple-Smith 2003). A recent success story is that of the western quoll, or chuditch (*Dasyurus geoffroii*) which was once the most widely distributed quoll species (found across 70% of Australia), but populations had contracted and declined to dangerously low numbers by the early 1990s (Fletcher and Morris 2003; Morris *et al.* 2003). A combined and dedicated effort to

captive breeding, predator control, translocations and wild population management has had a remarkable outcome in re-establishing the species in western Australia. The chuditch has thrived in the wild to an extent anticipated to result in them being downgraded to a conservation dependent category (Morris *et al.* 2003).

The remarkable taxonomic diversity of wildlife is also manifest in differences in the ecology, biology and behaviour, both between and within Family-level taxons (Russell 1982; 1984; Lee and Cockburn 1985; Tyndale-Biscoe and Renfree 1987; Bronson 1989; Croft 2003; Wildt *et al.* 2003). Unravelling the intricacies of a species' breeding biology is necessary for guiding recovery programs, and where necessary, the development of assisted reproductive techniques (Howard *et al.* 2003; Temple-Smith 2003; Wildt *et al.* 2003). An understanding of reproductive biology is considered to be the highest priority for developing and implementing effective recovery programs, and provides the basis for a sound and integrated approach to effective captive and wild population management (Hinds and Selwood 1990; Olney *et al.* 1994; Temple-Smith 2003).

Unfortunately, fundamental information on reproductive biology is often unavailable when required for concerted conservation efforts, highlighting the need for further investigations and progressive data collection on less common species (Temple-Smith 2003; Wildt *et al.* 2003). A prime example of this was the major gaps in our basic understanding of the breeding biology of devils and spotted-tailed quolls (both considered to be relatively 'safe' species at the onset of this study (Maxwell *et al.* 1996); with basic features such as age at maturity, the nature of the oestrous cycle, reproductive patterns and seasonality either unavailable or inferred at that time. Knowledge of reproductive biology of these two species is now urgently required to assist in improved management and recovery attempts (Jones *et al.* 2003; Hawkins *et al.* 2006). The present study has contributed through the development and validation of contemporary and practical techniques for monitoring reproduction that will assist in improved management of *in situ* and *ex situ* populations of devils and spotted-tailed quolls; these methods also have potential for extending our knowledge of other marsupial species.

Hormones are the "essence of reproduction" (Brown 2006), thus an understanding of endocrinology is invaluable to conservation efforts. A systematic analysis of male and female reproduction involving captive and wild populations will provide the level of detail necessary to guide meaningful management actions (Monfort 2003; Temple-Smith 2003; Wildt *et al.* 2003). Information gained through more traditional methods such as blood sampling have been steadily enhanced by the increasing application of non-invasive techniques, permitting development of more comprehensive databases to assist species management (reviews in: Lasley and Kirkpatrick 1991; Schwarzenberger *et al.* 1996).

Non-invasive monitoring of oestrous cycles, such as urinary and faecal steroid measurement provides an attractive alternative option to blood sampling, because it avoids any physiological changes associated with capture and physical handling and restraint (Lasley and Kirkpatrick 1991; Schwarzenberger *et al.* 1996). Faecal monitoring has not been widely applied to marsupials, but there is a growing body of literature suggesting its potential is recognised. Other studies have also applied this technique for characterising ovarian cycles or investigating the seasonality of breeding in several diprotodont and polyprotodont species (Hamilton *et al.* 2000; Stead-Richardson *et al.* 2001; Paris *et al.* 2002; Bradshaw *et al.* 2004; Oates *et al.* 2004; Woodd *et al.* 2006). This can be a particularly effective method for less tractable species, and for smaller species (*e.g.* honey possum <10g, Bradshaw *et al.* 2004) circumvents the need for capture or for collection of relatively considerable volumes of blood for assay.

Here, faecal steroid monitoring was successfully applied to measure reproductive activity in captive and free-ranging devils and spotted-tailed quolls. Particular benefits included enhanced, detailed information on male and female endocrine patterns resulting from increased sampling frequency, and longitudinal profiling of individuals throughout the year. This was especially useful for characterising phases of the ovarian cycle, given the typically brief duration of oestrus activity and the abbreviated luteal phase in marsupials (Tyndale-Biscoe and Renfree 1987). This technique is mainly relevant to reproductive monitoring of captive populations, and can assist in diagnosing causes of breeding failure; such as acyclic or irregular oestrous patterns in females. For

marsupials, the brevity of oestrus (Tyndale-Biscoe and Renfree 1987) and the time taken to perform plasma or faecal hormone analyses does place limitations on the use of this technology for breeding manipulation or management. Other methods of detecting oestrus - such as monitoring changes in vaginal cytology or body mass (in small dasyurids) offer less specific information, but provide an immediate result; therefore, this method has a more practical application for example, in determining appropriate timing of mating introductions. However, although endocrinology and vaginal cytology are useful tools for detecting oestrus and monitoring the oestrous cycle, both techniques still require a level of expertise and the use of analytical equipment/access to facilities.

The present research has demonstrated that pouch appearance is a simple, reliable and less invasive method for monitoring reproductive status in the devil and spotted-tailed quoll. Pouch appearance provides an instantaneous indication of the stage of the oestrous cycle; therefore, this method can be used as a tool for captive breeding management in these species. Pouch condition can be used to monitor key reproductive events such as onset of puberty and oestrus, and to determine when ovulation has occurred; this has strong value for captive breeding management and also for improved monitoring of wild populations.

A range of other marsupials exhibit characteristic changes in pouch morphology during the breeding season, similar to those described here for the devil and spotted-tailed quoll (Bollinger and Carrodus 1938; 1939b; 1939a; Woolley 1974; Jackson 2003b; Jackson *et al.* 2003; Power and Monoghan 2003; Finlayson *et al.* 2006). These common traits suggest that pouch appearance may also prove to be a useful indicator for monitoring reproductive condition in other species. However, as for the present study, this method first requires validation through physiological techniques, and an index of pouch condition/scoring will need to be generated for each species.

Knowledge of the reproductive biology of devils and spotted-tailed quolls can also contribute on a broader level to conservation efforts through providing information for improved management of wild populations. Understanding the impact of threatening factors requires, firstly, comprehensive information on the biology and ecology of the target species and, secondly, detailed monitoring so that any effect of perturbations can be gauged and management decisions guided accordingly (Jones *et al.* 2003; Temple-

Smith 2003). In dasyurids, including the spotted-tailed quoll and the devil, research findings can contribute to the evaluation and mitigation of the effects of proposed changes in land practice, assist with reserve planning; as well as reviewing pest control practices, such as the impact of poison baits on non-target species (Soderquist and Serena 1994; Hesterman and Jones 2001; Jones *et al.* 2003; Morris *et al.* 2003; Temple-Smith 2003; Wilson *et al.* 2003; Belcher and Darrant 2004; Körtner *et al.* 2004; Claridge *et al.* 2005; Glen and Dickman 2006).

7.4 Conclusions

Ultimately, this study has served as a timely reminder that fundamental knowledge of the reproductive biology of species we consider to be secure today may come too late when rapid stochastic events such as disease strike, and cause massive, rapid population losses in the wild (Hawkins *et al.* 2006). Trying to gather this information afterward may be an impossible task, and cannot necessarily be extrapolated from analogue species because of intricacies that exist within Families. As demonstrated here these differences may be quite profound, even between closely-related species. Study of *ex situ* populations (where these exist) provides valuable longitudinal data under controlled conditions for more detailed information on reproductive processes of a species. However, this does not necessarily inform us of the natural breeding biology of their wild counterparts – which is central to developing better management practices; and toward an ultimate goal to ensure species' preservation so they can fulfil their role in the ecosystem.

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APPENDIX A

Details of female study animals. Age and breeding status in 2001 are given, except where indicated.

Studbook / ID #	House Name	Age (Years)	Origin	Dam ID (where relevant)	Proven breeder?
Tasmanian Devils					
130	Maradunna	7	captive		yes
202	Moriarty	6	wild		yes
201	Moggle	6	captive		no
277	Moschka	5*	captive	#202	yes
273	Eumarrah	4*	wild		yes
274	Myrtle	3	captive	#130	no
330	Kimberley	2	wild		immature
328	Montana	2	wild		immature
331	Maloolah	2	wild		immature
296	Myalla	2	captive	#277	immature
304	Mara	2	captive	#273	immature
298	Manganinnee	2	captive	#277	immature
341	Dolly	1	wild		immature
-	Meara	1	captive	#273	immature
Spotted-tailed Quolls					
STQ #1	Donna	3*	wild		no
STQ #2	Loyetea	2	wild		yes
STQ #3	Letitia	2	wild		yes
STQ #4	Lemonthyme	1	wild	Letitia	immature
STQ #5	Luina	1	wild	Letitia	immature
STQ #6	Lilico	1	wild	Letitia	immature
STQ #7	Liena	1	wild	Letitia	immature

* also included in 2000

Note: Three additional females from FWP sampled to provide supplemental data (2001) not included in summary

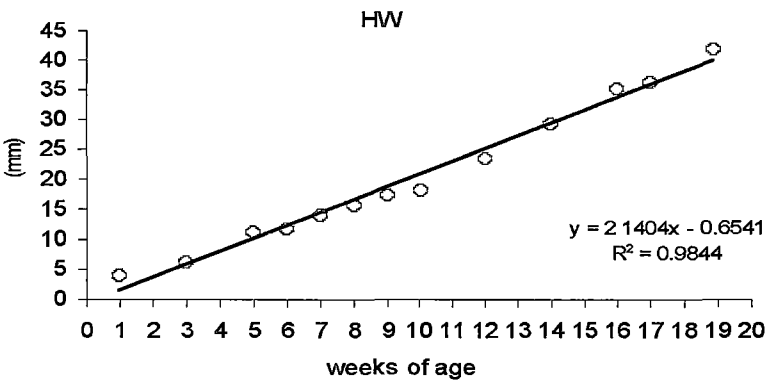
APPENDIX B

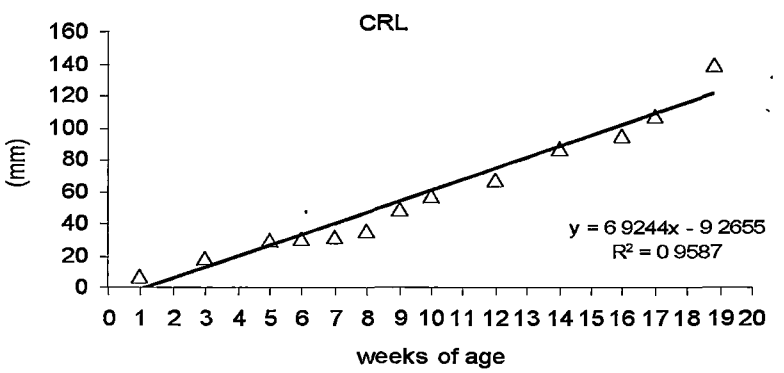
Details of male study animals. Age and breeding status at the onset of 2001 are given, except where indicated.

Studbook / ID #	House Name	Age (Years)	Origin	Dam ID (where relevant)
Tasmanian Devils				
126	Clem	7	wild	
203	Meander	5	captive	
272	Murchisson	4	captive	
295	Manna	2	captive	#277
329	Maudey	2	wild	
666	Sydney	4	captive	
Spotted-tailed Quolls				
STQ #11	CJ	3	captive	
STQ #12	Loongana	2	wild	
STQ #13	Dog's Head	2	wild	
STQ #14	Lorin	1	wild	
STQ #15	Lemana	1	wild	
STQ #16	Whitefoot	1	wild	#2
STQ #17	Leith	1	wild	#2
STQ #18	Luna	1	wild	#3

APPENDIX C

Growth curves constructed from average measurements (mm) for captive Tasmanian devil pouch young (n = 6 litters); HW = head width, CRL = crown-rump length.





APPENDIX D

Median number of Tasmanian devil pouch young for females in different age classes at Freycinet NP (FNP 1999 – 2001) and Meander district (MM 2001). Chi-square value indicated below each group ($P > 0.05$ for all cases).

Age	FNP			MM
	1999	2000	2001	2001
1	4.0	2.5	4.0	4.0
2	4.0	4.0	4.0	3.5
3	3.5	4.0	4.0	4.0
4	4.0	3.5	4.0	3.0
5	4.0	-	2.0	-
Total Litters	25	39	21	22
X^2 (*d.f.* = 3)	P = 1 00	P = 0.33	P = 0.91	P = 0.37